**NOTE**  Pathology

**Immunohistochemical Study of Osteopontin in the Spinal Cords of Rats with Clip Compression Injury**

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**ABSTRACT.** Expression of osteopontin (OPN) was investigated in the spinal cords of rats with clip compression injury. Western blot analysis demonstrated that OPN protein increased significantly in the spinal cord during the early stages after injury. The increased expression of OPN was partially paralleled by that of proliferating cell nuclear antigen (PCNA). Immunohistochemical staining showed that OPN was expressed in proliferating activated microglia/macrophages in core lesions and in some astrocytes at the periphery of lesions. These results indicate that expression of OPN protein increases mainly in activated microglia/macrophages after spinal cord injury, suggesting that OPN is related to cell proliferation during the early stages after injury, probably leading to tissue remodeling.

**KEY WORDS:** macrophage, osteopontin, spinal cord injury.

Spinal cord injury (SCI) is postulated to include primary mechanical injury and secondary damage that is induced by inflammatory responses. The neuropathological outcome of SCI is characterized by edema, axonal degeneration, infiltration of inflammatory cells, and reactive astrogliosis, in both clip compression injury and weight-drop contusion models [1]. In the process of SCI, cellular infiltration, proliferation of macrophages and astrogliosis occur at the site of injury [7].

Osteopontin (OPN) is an integrin- and calcium-binding phosphoprotein that is produced by mineralized tissue cells, many epithelial cells, and activated immune system cells [2]. OPN production is increased in several pathological conditions, including nephritis [5] and atherosclerosis [3]. The expression of OPN in pathological conditions of the central nervous system remains poorly understood. Studies of neuronal tissue have shown that OPN mRNA is expressed in neurons of the rat central nervous system [11] and is upregulated in microglia upon kainic acid-induced brain injury [8] and spinal cord injury [4]. In addition, the phosphorylation-dependent interactions of OPN with its receptors regulate macrophage migration and activation [14, 15]. Recently, it was reported that OPN mRNA and protein were detected in the injured spinal cord at 3 days post-injury, and that the cells expressing OPN were activated microglia [4]. However, little is known about the quantitative changes in OPN protein or the possible contribution of OPN to the activities of other molecules, including proliferating cell nuclear antigen (PCNA), in the course of spinal cord degeneration or repair. In our previous study, we found that phospholipase D1 is expressed after spinal cord injury, and postulated that it plays an important role in the proliferation of macrophages after spinal cord injury [7]. In the present study, we examined the pattern of OPN expression in rat spinal cord injury and the relationship between the expression of OPN and PCNA.

Sprague-Dawley rats were purchased from Daehan Biolink Co. (Chungbuk, Korea) and bred in our animal facility. Male rats weighing 160–200 grams, 7–12 weeks old, were used throughout the experiments. All experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

The surgical procedure to produce clip compression injury was performed using a modification of a previously published method [12]. The animals were anesthetized and subjected to laminectomy at T9/T10. Immediately following laminectomy, the spinal cord was compressed with a vascular clip (Stoelting, Wood Dale, IL, U.S.A.) that was applied vertically to the exposed spinal cord at an occlusion pressure of 15–20 g for 1 min. After compression, the muscles and skin layers were closed. In sham-operated control rats, only the laminectomy was performed. A total of 18 rats were used in this study. After injury to the spinal cord, and at the time tissues were taken, the animals lacked all hind limb locomotion. The animals were sacrificed on day 0 (sham-operated controls, n=3), and on post-injury days 1 (n=5), 4 (n=5) and 7 (n=5). Spinal cord tissues from the surgical sites were harvested and fixed or frozen for histological and Western blot analyses, respectively.

Frozen spinal cords were thawed at room temperature, minced, lysed in a lysis buffer (20 mM HEPES, pH 7.2, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 10 µg/ml leupeptin, 10 µg/ml aprotonin, and 1 mM phenylmethylsulfonyl fluoride), and homogenized. Samples were electrophoresed under denaturing conditions by 7.5% SDS-
PAGE and then blotted onto PVDF membranes. Each blot was probed with monoclonal anti-OPN antibody (Akm2A1, sc-21742, IgG 1; 1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or mouse monoclonal anti-PCNA (1:1,000 dilution; Sigma, St. Louis, MO, U.S.A.), each diluted in blocking solution. The immunoblot membranes were reprobed with a monoclonal antibody against beta-actin (Sigma). After incubating the blot with appropriate horseradish peroxidase-conjugated secondary antibody (Vector, Burlingame, CA, U.S.A.), visualization of binding was achieved using Amersham ECL reagents (Amersham, Arlington Heights, IL, U.S.A.). The results were quantified with a densitometer (M GS-700 Imaging Densitometer, Bio-Rad Laboratories, Hercules, CA). The relative expression of OPN and PCNA were calculated after normalization to beta-actin from three different samples. The results were further analyzed among groups (n=3) using the post-hoc Student-Newman-Keuls’ procedure for multiple comparisons.

Paraffin-embedded spinal cords were sectioned (5-µm) and deparaffinized before the samples were allowed to react with primary antisera, including monoclonal anti-OPN antibody (1:800 dilution) and mouse monoclonal anti-PCNA (1:1,600 dilution). Anti-PCNA antibody was used to demonstrate proliferative activity. To identify astrocytes and macrophages, rabbit anti-glia fibrillary acidic protein (GFAP; 1:800 dilution; Dako, Copenhagen, Denmark) and mouse monoclonal anti-rat activated macrophages (ED1; 1:800 dilution; Serotec, London, UK) were applied, respectively. To visualize OPN-expressing glial cells and macrophages in spinal cord lesions, adjacent serial sections were reacted with antibodies to OPN, GFAP, or ED1. The immunoreactivity was visualized as an avidin-biotin peroxidase complex with an Elite kit (Vector). The peroxidase reaction was developed with a diaminobenzidine substrate kit (Vector).

To examine the expression pattern of OPN, Western blot analysis was performed on the injured spinal cord tissues. Figure 1 shows two OPN bands of approximately 60 and 30 kDa, in agreement with a previous study by Luedtke et al. [10]. Expression of OPN was low in normal spinal cord (lane 1) and gradually increased on post-injury days 1 (lane 2), 4 (lane 3) and 7 (lane 4). The significant increase of OPN expression started after day 1 (Fig. 1, lane 2)(Density OD/mm² value [mean ± SD], upper band: 1 ± 0.1 [p<0.05], lower band: 0.3 ± 0.1), and there was a significant peak on day 4 (Fig. 1, lane 3)(upper band: 1.6 ± 0.3 [p<0.01], lower band: 0.6 ± 0.2) and 7 (Fig. 1, lane 4)(upper band: 1.5 ± 0.5 [p<0.01], lower band: 0.7 ± 0.2; [p<0.05]), as compared to sham-operated controls (Fig. 1, lane 1)(upper band: 0.4 ± 0.1, lower band: 0.3 ± 0.1). In Fig. 2, the expression of PCNA was detected weakly in sham-operated controls (lane 1, 4.9 ± 0.1), and significantly increased after injury through day 1 (lane 2, 7.75 ± 0.4, p<0.01), 4 (lane 3, 8.4 ± 0.6, p<0.01) and 7 (lane 4, 6.9 ± 0.3, p<0.01), indicating that the increased expression of PCNA and OPN temporarily over-

![Fig. 1. Western blot analysis of OPN and beta-actin at day 0 (sham-operated control, lane 1), and post-injury days 1 (lane 2), 4 (lane 3) and 7 (lane 4). A representative photograph of Western blot analyses of OPN and beta-actin. Two weak OPN bands were present in the sham-operated control sample (lane 1). The intensity of the OPN bands increased in samples from post-injury days 1 (lane 2), 4 (lane 3) and 7 (lane 4). The approximate molecular weight of two bands was 60 and 30 kDa. The relative expression of OPN was calculated after normalization to beta-actin from three different samples.](image1)

![Fig. 2. There was an increase in PCNA on day 0 (sham-operated control, lane 1), and post-injury days 1 (lane 2), 4 (lane 3) and 7 (lane 4). The molecular mass of PCNA is 36 kDa. The relative expression of PCNA was calculated after normalization to beta-actin from three different samples.](image2)

lapped in the early stage of spinal cord injury.

All histological examinations in this study showed similar findings, which were largely consistent with our previous report [6]. In the spinal cords of normal or sham-operated rats, in which no pathological lesions were seen, weak immunostaining for OPN was detected in some neurons and some astrocytes (Fig. 3A). At 1 day post-injury, some OPN-positive cells were detected in the edematous and disorganized regions of the white and gray matter of the injured spinal cords (Fig. 3B). Four days post-injury, OPN immunoreactivity was increased in the infiltrating round cells and the activated microglia in the lesions (Fig. 3C). At day 7 post-injury, a majority of the cells in the damage cavity expressed OPN (Fig. 3D), and those cells were also positive for ED1 (Fig. 3F). OPN was also detected in some glial cells, identical to astrocytes, in the periphery of the
In a previous study, we reported that the majority of macrophages were positive for PCNA at day 4 post-injury [6]. To examine distribution and co-localization of OPN and PCNA in the injured spinal cord, adjacent serial sections were immunostained for either OPN or PCNA. The majority of OPN-positive cells (Fig. 4A) were also PCNA-positive (Fig. 4B). Arrows indicate the same cells in A and B. A and B: counterstained with hematoxylin. Bar represents 30 µm.

Fig. 4. Immunostaining of OPN and PCNA on day 7 post-injury in adjacent serial sections. The majority of OPN-positive cells (A) were also PCNA-positive (B). Arrows indicate the same cells in A and B. A and B: counterstained with hematoxylin. Bar represents 30 µm.

In the present study, we report that the expression of OPN is quantitatively increased after spinal cord injury, and that the increased OPN expression is partially related to the expression of PCNA. Moreover, it was shown that the majority of proliferating cells in the early stage of spinal cord injury express OPN. It is postulated that OPN plays an important role in mediating the inflammatory process during the early stages of spinal cord injury, and that OPN is produced in a variety of cell types, including activated microglia/macrophages, as well as astrocytes and neurons in damaged tissues.

OPN is known to facilitate the migration of inflammatory cells and glial cells into the damage cavity after injury, and is deeply involved in cell proliferation [16]. The adhesive and migratory effects of OPN are mediated via distinct cell surface integrins, and OPN may promote cell attachment and mediate cell migration [9]. OPN was found to be involved in the migration of C6 glioma cells in vitro studies [13]. Considering the above findings, we postulate that OPN facilitates cell migration of macrophages and astrocytes after spinal cord injury.

It is interesting to consider the role of OPN in cell proliferation. The expression of OPN is up-regulated in PCNA-positive cells in nephritis [16], similar to our finding of the co-localization of OPN and PCNA in proliferating cells in the early stage of spinal cord injury. In this study, we found that an increase in PCNA and OPN was seen in the spinal cords of rats after SCI, and that OPN expression increased in activated microglia/macrophages and in some glial cells, while neuronal OPN did not change. This leads us to propose that cell proliferation is intimately associated with OPN expression, as either a cause or a consequence.

Some studies have reported that OPN mRNA expression temporarily peaks at day 5 in focal stroke [13] and at day 3 after spinal cord injury [4]. In the present study, we also found a significant increase in OPN in the early stage (days 1 to 7) of SCI. The immunohistochemistry findings of OPN expression over the course of SCI paralleled the results of the Western blot analysis. Our results were based on a severe spinal cord injury and thus may differ slightly from the results of Hashimoto et al. [4], who used a relatively mild injury, in which the majority of OPN-positive cells were activated microglia. Moreover, Fu et al. [6] have reported that a tendency toward increased OPN immunore-
activity was seen in glial cells, neurons, and activated microglia/macrophages following spinal root avulsion. The authors suggested that OPN has two distinct functions in the CNS: one in the inflammatory response, mediated by glial cells and possibly neurons, and the other in the protection of neurons from iNOS-mediated degeneration.

Considering all of these findings, it is postulated that OPN protein is increased in the course of spinal cord injury and is possibly involved in cell proliferation, the facilitation of cell migration, and finally the remodeling of damaged spinal cord. The elucidation of the precise mechanisms of OPN actions using OPN knockout mice requires further study.

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REFERENCES