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Different expression of macrophages and microglia in rat spinal cord contusion injury model at morphological and regional levels.

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Abstract

Macrophages and microglia are implicated in spinal cord injury, but their precise role is not clear. In the present study, activation of these cells was examined in a spinal cord injury model using 2 different antibodies against ED1 clone and ionized calcium binding adaptor molecule 1 (Iba1). Activation was observed at 1, 4, 8, and 12 weeks after contusion injury and was compared with sham operated controls. Our results indicate that activation could be observed in both the dorsal funiculus and the ventral white matter area in the spinal cord at 5 mm rostral to the epicenter of injury. For both cells, there was a gradual increase in activation from 1-4 weeks, followed by down-regulation for up to 12 weeks. As a result, we could stain macrophages by ED1 and microglia by Iba1. We concluded that macrophages may play a role in the phagocytosis of denatured dendrites after spinal cord injury, while microglia may have some cooperative functions, as they were found scattered near the macrophages.

KEYWORDS: macrophages, microglia, spinal cord injury, ED1, Iba1

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Original Article

Different Expression of Macrophages and Microglia in Rat Spinal Cord Contusion Injury Model at Morphological and Regional Levels

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Macrophages and microglia are implicated in spinal cord injury, but their precise role is not clear. In the present study, activation of these cells was examined in a spinal cord injury model using 2 different antibodies against ED1 clone and ionized calcium binding adaptor molecule 1 (Iba1). Activation was observed at 1, 4, 8, and 12 weeks after contusion injury and was compared with sham operated controls. Our results indicate that activation could be observed in both the dorsal funiculus and the ventral white matter area in the spinal cord at 5 mm rostral to the epicenter of injury. For both cells, there was a gradual increase in activation from 1-4 weeks, followed by down-regulation for up to 12 weeks. As a result, we could stain macrophages by ED1 and microglia by Iba1. We concluded that macrophages may play a role in the phagocytosis of denatured dendrites after spinal cord injury, while microglia may have some cooperative functions, as they were found scattered near the macrophages.

Key words: macrophages, microglia, spinal cord injury, ED1, Iba1

Applying sufficient weight to the spinal cord can induce complete or incomplete paralysis [1, 2]. The possible causes of the induced paralysis are related to many factors and changes and may occur in 2 stages: primary injury due to tissue detrition and necrosis as a result of external mechanical forces; and secondary injury caused by ischemia [3], excitoaminotoxicity [4], and apoptosis [5]. One of the changes occurring after spinal cord injury (SCI) is the activation of microglia and macrophages [6]. Microglia are thought to contribute to the onset of or to exacerbate neuronal degeneration and/or inflammation in many brain diseases by producing deleterious substances such as superoxide anions, nitric oxide, and inflammatory cytokines [7, 8]. However, another function of microglia and macrophages is thought to be brain defense, as they are known to scavenge invading microorganisms and dead cells, and also to act as immune or immunoeffector cells [9]. Moreover, microglia produce neurotrophic and/or neuroprotective molecules, and it has been suggested that they promote neuronal survival in cases of brain injury [10]. However, the precise role of macrophages and/or microglia as neurotoxic cells or as neuroprotective cells *in vivo* remains unclear.

The objective of the present study was to examine the activation of macrophages and microglia after spinal cord injury using 2 different types of antibodies for these cells.

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Materials and Methods

Operative Procedure and Tissue Preparation [11, 12]. Male Sprague-Dawley rats (n = 25) with an average body weight of 312 g (280 to 330 g) were used to prepare the model of contusive traumatic SCI. After anesthetizing the animals by administering pentobarbital sodium intraperitoneally (30 mg/kg), the animals were fixed on a surgical table and surgery was performed. A midline skin incision was made on the dorsal side, and the spinal column was exposed from the T9 to L1 levels. The lamina of T11 and T12 were carefully removed using microrongeurs, starting from the caudal edge of the T12 lamina. After laminectomy, the dura was confirmed to be intact. The clamp was adjusted and fixed to ensure that the spinal column was horizontal relative to the floor. In the experimental group (SCI group: n = 20), a metal rod 2 mm in diameter weighing 30 g was gently placed centrally onto the dura of the spinal cord on the dorsal side. Compression was then applied continuously for 10 min to produce the contusive injury model. In sham-operated animals (control group: n = 5), the same dural exposure procedure was performed, but the spinal cord was not compressed. During the surgical procedure, body temperature was controlled at 37 °C using a feedback-controlled heating pad (Temperature controller; CMA, Stockholm, Sweden) and by rectal temperature monitoring. Twenty minutes after the end of compression, all rats were housed under a 12-h light regime, with one animal per cage. Water and food were provided ad libitum. Bladders were emptied manually twice daily until sufficient bladder-emptying function returned. All experimental procedures were performed in compliance with guidelines for the care and use of animals set forth by the American Journal of Physiology and those established by the Institute of Laboratory Animal Sciences of the Faculty of Medicine of Kagawa University.

SCI group animals were sacrificed at 1, 4, 8, and 12 weeks (n = 5, for each stage), respectively, after surgery. The animals were anesthetized by administering pentobarbital sodium intraperitoneally, and then perfused transcardially with phosphate-buffered saline (PBS) and perfusion-fixed with 4% neutral buffered formalin solution. The spinal cord in the vicinity of the injury was immediately removed and dissected. A segment approximately 5 mm rostral to the injury site was cut as a block and post-fixed in the above formalin solution for 48 h. After successive treatment with 10–30% sucrose solutions, the blocks were embedded in OCT compound. Frozen 20-μm cross-sections at 5 mm rostral were then prepared and examined.

Immunohistochemistry. For the immunohistological examination, frozen sections were dried completely and immersed in 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) in 0.01 M PBS for 1 h. To quench endogenous peroxidase activity, the sections were immersed in 2% H_2O_2 in 0.01 M PBS for 30 min. After washing with 0.01 M PBS, nonspecific reactions were blocked with 1% albumin solution. The monoclonal antibody ED1 from BMA Biomedicals AG (August, Switzerland) was used as a marker for rat macrophages as the primary antibody [13]. Polyclonal antibody Iba1 (ionized calcium-binding adaptor molecule 1), which has been found to be highly and specifically expressed in microglia in both *in vivo* and *in vitro* studies, was obtained from Wako chemicals (Tokyo, Japan) [14, 15]. Some amount of Iba1 antibody was donated by Dr. S. Kohsaka of the Department of Neurochemistry, National Institute of Neuroscience, Kodaira, Tokyo, Japan. Both ED1 and Iba1 antibodies have been reported to recognize macrophages and/or microglia [13, 15]. We used them for the detection of activated macrophages and microglia. The primary antibody was first diluted 1:800 for ED1 and 1:1000 for Iba1 in 1% albumin solution in 0.01 M PBS, and then applied to sections overnight at 4 °C. The sections were then incubated with a biotinylated antibody for another hour and an avidin-biotin peroxidase complex for 1 h (ABC Kit; Vector laboratories, Burlingame, CA, USA). The final peroxidase reaction was visualized using a solution of 3, 3'-diaminobenzidine in 0.03% H_2O_2 in 0.01 M PBS.

Double-labeling immunohistochemistry of frozen sections was performed using the above-described procedure up to blocking of the reactions with 1% albumin solution. Sections were then incubated for 24 h at 4 °C with anti-ED1 monoclonal antibody and anti-Iba1 polyclonal antibody diluted to 1:800 and 1:1000, respectively, in 1 % albumin solution in 0.01 M PBS. After washing with 0.01 M PBS, the ED1 and Iba1 reactions were carried out by incubation for 2 h at room temperature with anti-mouse IgG conjugated with Texas Red-X (Molecular Probes, Eugene, OR, USA) and anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Vector), both diluted to 1:100 in 1% albumin solution in 0.01 M PBS. Finally, the sections were rinsed in 0.01 M PBS and visualized using a confocal laser scanning microscope

(LSM-GB200; OLYMPUS, Tokyo, Japan).

Triple immunohistochemistry staining was performed using the above-described procedure up to incubating the sections with fluorescence-conjugated secondary antibodies. After washing with 0.01 M PBS, the sections were incubated for 20 min in DAPI solution (Molecular Probes) at room temperature, which was diluted to 400 nM in 1% albumin solution in 0.01 M PBS. Finally, the sections were rinsed in 0.01 M PBS and visualized using a confocal laser scanning microscope (Bio-Rad Radiance2100, Tokyo, Japan).

Data analysis. Image analysis was performed with Image-Pro Plus software (version 4.0, Media Cybernetics, The Imaging Expert, Silver Spring, MD, USA) to assess the altered immunostained area density of ED1 antigen and Iba1 expression (area μm^2) 5 mm rostral to the injury site. Cross sections were examined under a light microscope at 400× magnification. Photographs of the area of dorsal funiculus and ventral white matter were taken and used for analysis. For evaluation of expression in white matter, the average of values from the 2 sides was used in statistical analysis. A total of 50 photographs of the white matter and 25 of the dorsal funiculus were prepared. Semi-quantitative evaluation by Image-Pro Plus analysis of photographs was performed blindly by 2 researchers not directly involved in the present study. To evaluate differences in the area of immunoreactive ED1 and Iba1 expression in the dorsal funiculus and ventral white matter, one-way analysis of variance (ANOVA) and Scheffe's F-test were performed. Differences in expression between time points and regions were evaluated. In all statistical analyses, differences with a P value of < 0.01 were considered statistically significant.

Results

A weak ED1 immunoreactivity (ir) was detected in the white matter, including the dorsal funiculus of sham-operated rat (control group) (Fig. 1A). Ramified and fibrous shapes of microglia were observed (Fig. 2A). The ir increased gradually after contusion (SCI group) (Fig. 3A). Strong activation of ED1 ir at 4 weeks after contusion in both the ventral white matter and the dorsal funiculus was observed (Fig. 1B). Many ED1-positive cells became round in shape with granules in the cytoplasm (Fig. 2B). This tendency became clearer at 8 weeks after SCI (Fig. 1 C and 2 C). At 12 weeks, a down-regulation of expression was observed (Fig. 3A). In contrast, Iba1 ir was detected throughout the white matter and gray matter in sham-operated rats (Fig. 1D). Iba1-positive cells showed ramified and short rod shapes with the morphology of resting microglia (Fig. 2D). The ir of Iba1 also increased gradually, similar to that of ED1 (Fig. 3B). At the dorsal funiculus, Iba1-positive cells increased in number and ir at 4 weeks after contusion (Fig. 1E). This tendency became clearer at 8 weeks after injury (Fig. 1F and 2F). At 12 weeks, down-regulation was also observed (Fig. 3B). These cells had heterogeneous shapes (data not shown), but the ir increased clearly in comparison with normal cells. Ibal-positive cells showed 2 different morphologies: cells with round or ameboid cell bodies with no apparent granules in the cytoplasm, or ramified cells with small cell bodies (Fig. 2E). At 8 weeks, many round cells were stained, with some cells existing around small cisterns (Fig. 2F). The same tendency was observed in the ventral white matter (data not shown).

Fig. 4 shows double staining with ED1 and Iba1. At 4 weeks after SCI, many big, round ED1-positive cells with phagocytic morphology were detected at the site of the dorsal funiculus (Fig. 4B). At the same time, some Iba1-positive cells were also detected with phagocytic morphology; while some Iba1-positive cells showed small cell bodies with processes (Fig. 4A). We detected the co-expression of ED1 and Iba1 in some cells, while some cells were ED1-positive but Iba1-negative, or Iba1-positive but ED1-negative (Fig. 4 C). These results can be clearly seen in Fig. 5, which shows ED1-positive macrophages and Iba1-positive microglia existing separately (Fig. 5D). Meanwhile, at 4 weeks after SCI, Iba1-positive cells were found distributed widely in the gray matter, while no ED1-positive cells could be found (Fig. 4, D-F). Different from the phagocytic morphology in the dorsal funiculus at the same time point, Iba1-positive cells in the gray matter showed small cell bodies with ramified processes, which reflected the morphology of the microglia (Fig. 4D, F). Overall, although ED1- and Iba1-positive cells stained differently, they occurred in the vicinity of each other (Fig. 4 C).

Discussion

In the present study we were able to stain ED1- and Iba1-positive cells differentially in the experimental model of SCI. Many of the ED1-positive cells were large and multivaluolated, consistent with the morphology of

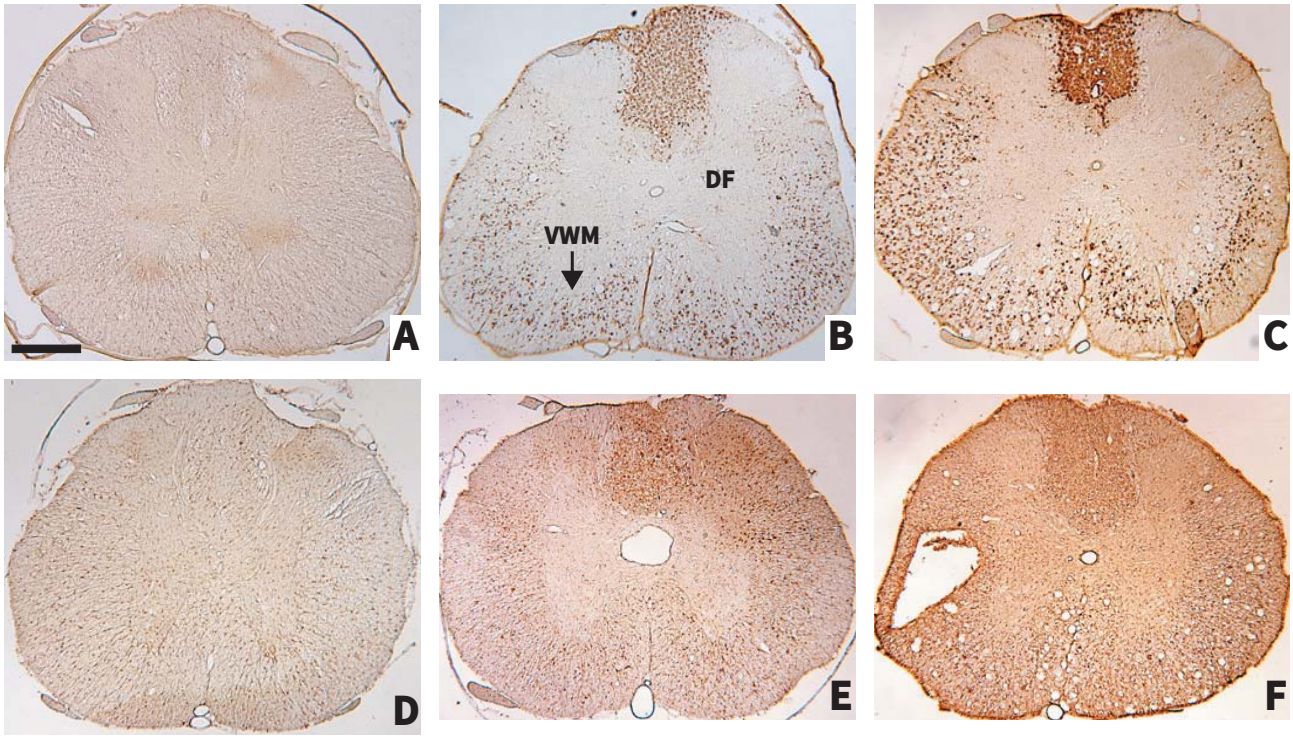

Fig. 1 Legend on the opposite page.

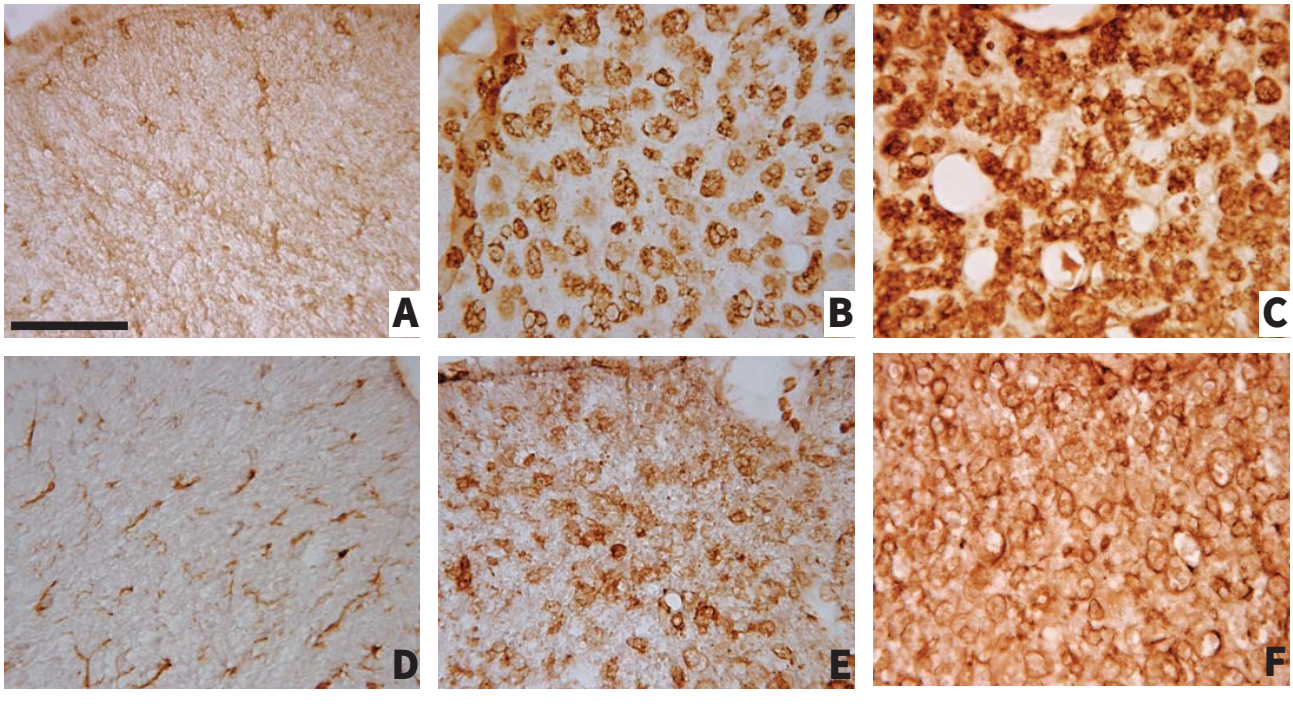

Fig. 2 Legend on the opposite page.

Fig. 1 Immunohistochemical staining for ED1 and Iba1 after spinal cord injury at low magnification. The upper column shows ED1 staining; the lower column shows Iba1 staining. **A**, **D** (normal); **B**, **E** (4 weeks after injury); **C**, **F** (8 weeks after injury). Arrows show the activation sites. Scale bars: 500 μm. DF = dorsal funiculus; VWM = ventral white matter.

Fig. 2 Immunohistochemical staining for ED1 and Iba1 at the dorsal funiculus after spinal cord injury at higher magnification. The upper column shows ED1 staining; the lower column shows Iba1 staining. **A**, **D** (normal); **B**, **E** (4 weeks after injury); **C**, **F** (8 weeks after injury). Scale bars: 100 μm.

Fig. 3 Measured area of ED1 and Iba1 expression (μm^2) in the dorsal funiculus of a cross section of spinal cord before and after spinal cord injury. **A**, ED1; **B**, Iba1. Mean values of total antibody expression between the spinal cord injury and control group were compared at each time point for both antibodies. *, $P < 0.01$ compared with control by one-way ANOVA. Bars indicate group means (± SEM).

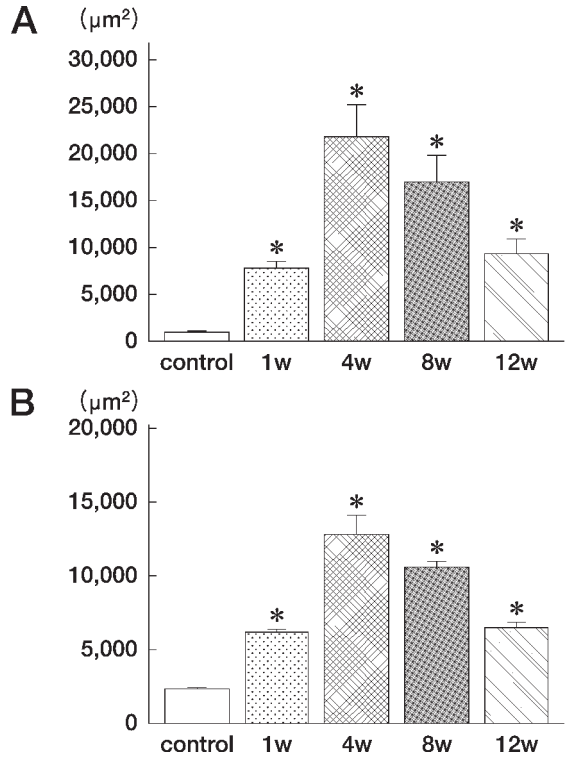

Fig. 3

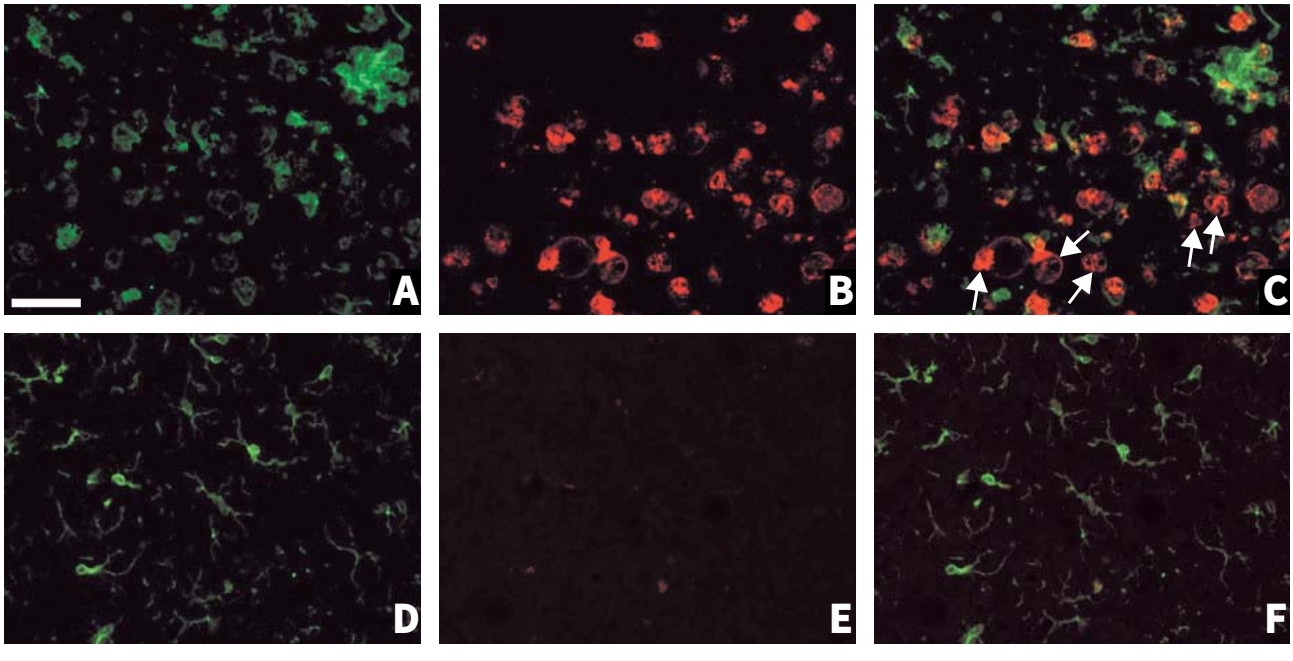

Fig. 4 Double immunohistochemical staining for ED1 and Iba1 in a cross section 5 mm rostral to the epicenter of injury at 4 weeks after injury, using confocal microscopy. **A**, **D**: Iba1 visualized with FITC; **B**, **E**: ED1 visualized with Texas Red; **C**, **F**: visualization of both ED1 and Iba1. Arrows show specific staining for ED1, arrow heads show colocalization. The upper column shows ventral white matter; the lower column shows gray matter. Scale bar: 50 μm.

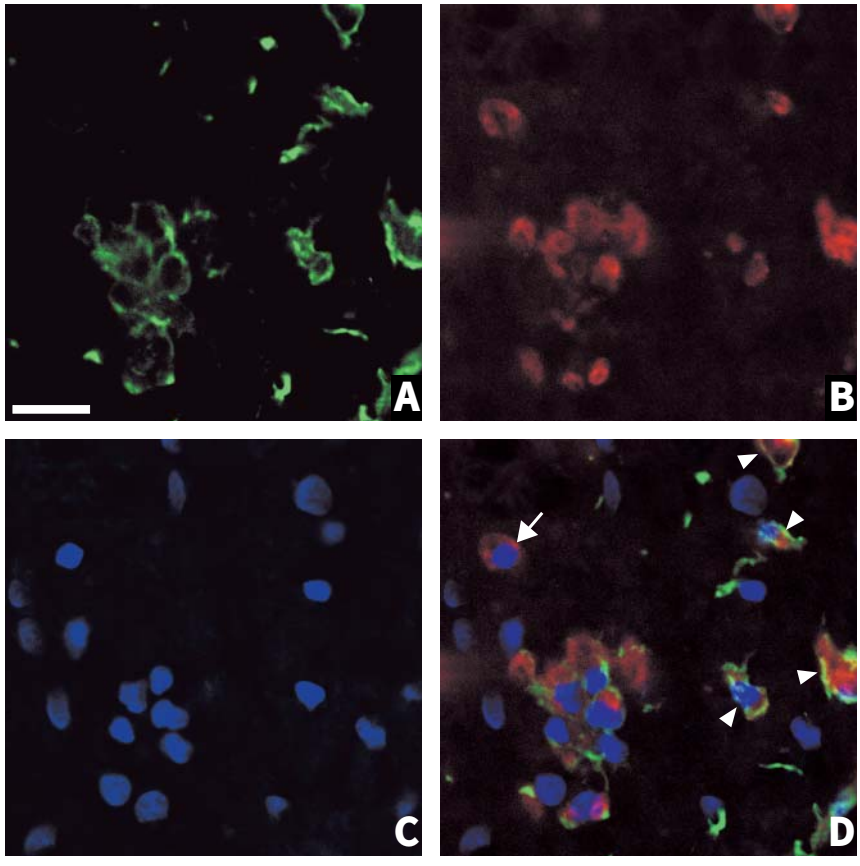

Fig. 5 Triple immunohistochemical staining for ED1, Iba1, and DAPI in a cross section 5 mm rostral to the epicenter of injury at 4 weeks after injury, using confocal microscopy. **A**, Iba1 visualized with FITC; **B**, ED1 visualized with Texas Red; **C**, nucleus visualized with DAPI; **D**, merging image of ED1, Iba1 and DAPI. An arrow indicates specific staining for ED1; an open arrow shows specific staining for Iba1; arrowheads show colocalization. Scale bar: 20 μm.

activated macrophages [16]. On the other hand, the Iba1-positive cells may have been microglia, judging from their shape and antigen specificity [6, 15], as has previously been reported [17]. However, in the previous study, a different brain-injury model called the middle artery occlusion model was used, and expression of ED1 antigen was found to be restricted to severe ischemic damage and activated microglia were observed in the peri-ischemic area [17]. Although some studies have reported that ED1 and Iba1 stain both microglia and macrophages [18–20], in the present study we separately detected activation of the microglia and macrophages. Our detection was based on the findings of Damoiseaux [13], who proved that monoclonal antibody (mAb) ED1 binds specifically to a single-chain glycoprotein expressed predominantly on the lysosomal membranes of activated macrophages, and on the findings of Ito and Imai, whose works indicate that Iba1 is expressed in microglia alone both in cultured brain cells and in the brain [14, 15]. On the other hand, although most studies have been carried out at the acute phase of SCI [16], our experiment was performed at the delayed phase. All these factors may have some influence on the activation of macrophages and microglia following SCI.

In the present study, the activation of microglia and macrophages was detected 4 weeks after injury. These regions are impaired easily [1, 12], possibly resulting in many vacuoles, as observed after 12 weeks in the present study. In response to central nervous system (CNS) injuries, activated microglial cells may transform from process-bearing (ramified) morphology to an amoeboid shape [21] and at times may present some phagocytic properties of macrophages. This result may explain the results of the double-staining, indicating that some Iba1-

positive cells were also ED1-positive, while others were ED1 negative. Macrophages may play a role in the phagocytosis of denatured cell debris [22]. Since in the present study microglia were found to be scattered near macrophages, some cooperative microglial role may be suggested. However, further studies are required to explore such a possibility.

In conclusion, in the present study we were able to separately stain macrophages and microglia following SCI. These cells were activated at 4 weeks and down-regulated around 12 weeks after injury. The shapes of the stained macrophages and microglia were different, but the regions in which these cells existed were the same.

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