Periostin Promotes Scar Formation through the Interaction between Pericytes and Infiltrating Monocytes/Macrophages after Spinal Cord Injury

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Accepted for publication November 22, 2016.

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Scal formation is a prominent pathological feature of traumatic central nervous system (CNS) injury, which has long been implicated as a major impediment to the CNS regeneration. However, the factors affecting such scar formation remain to be elucidated. We herein demonstrate that the extracellular matrix protein periostin (POSTN) is a key player in scar formation after traumatic spinal cord injury (SCI). Using high-throughput RNA sequencing data sets, we found that the genes involved in the extracellular region, such as POSTN, were significantly expressed in the injured spinal cord. The expression of POSTN peaked at 7 days after SCI, predominantly in the scar-forming pericytes. Notably, we found that genetic deletion of POSTN in mice reduced scar formation at the lesion site by suppressing the proliferation of the pericytes. Conversely, we found that recombinant POSTN promoted the migration capacity of the monocytes/macrophages and increased the expression of tumor necrosis factor-α from the monocytes/macrophages in vitro, which facilitated the proliferation of pericytes. Furthermore, we revealed that the pharmacological blockade of POSTN suppressed scar formation and improved the long-term functional outcome after SCI. Our findings suggest a potential mechanism whereby POSTN regulates the scar formation after SCI and provide significant evidence that POSTN is a promising therapeutic target for CNS injury. (Am J Pathol 2017, 187: 639–653; http://dx.doi.org/10.1016/j.ajpath.2016.11.010)

Traumatic spinal cord injury (SCI) causes permanent motor/sensory dysfunction, resulting in a marked reduction in the quality of life.1,2 Despite the recent advances in the pathophysiological understanding of SCI, there are few effective clinical treatments for SCI patients.3–5 Scar formation is a prominent pathological feature of SCI, which has long been implicated as a major impediment to the regeneration of the central nervous system (CNS).6,7 Spinal cord injuries in humans and experimental rodents are often associated with varying degrees of spontaneous functional recovery during the first month after injury.8,9 During the period after SCI, the progression of scar formation is observed in the injured spinal cord.10 Although scar formation after SCI is a well-known process, the factors involved in the scar formation remain to be elucidated.

Pericytes are known to be important in physiological contexts, including the support of vascular structure, maintenance of the blood-brain barrier, regulation of cerebral blood flow, and facilitation of vessel maturation.11–13 In addition to these activities, pericytes have recently been identified as a source of scar-forming cells in the injured spinal cord14; however, little is known about the physiological function of scar-forming pericytes.

Supported by Ministry of Education, Science, Sports, and Culture of Japan grant-in-aid for Scientific Research (B) (S.O.); Japan Society for the Promotion of Science Research Fellows grant 26-745 (K.Y.); and research foundations from the General Insurance Association of Japan (K.Y.).

Disclosures: None declared.
Periostin (POSTN) is an extracellular matrix protein belonging to the fasciclin family.\textsuperscript{15} POSTN plays an essential role in the development of bone, tooth, and heart tissue.\textsuperscript{16–18} The expression of POSTN is observed predominantly at the early stages of embryogenesis, with its expression down-regulated in adult tissues.\textsuperscript{19,20} In the present study, we detected significantly up-regulated expression of POSTN in injured spinal cords of adult mice using high-throughput mRNA screening. Several lines of evidence have suggested that POSTN is involved in fibrotic changes of various organs, including the liver, bronchus, lung, and skin.\textsuperscript{21–23} We therefore hypothesized that POSTN would be involved in the scar formation after SCI.

In this study, we used both genetic manipulation and a pharmacological approach to clarify the contribution of POSTN to scar formation and the functional outcome after SCI in mice. We found that POSTN up-regulated the expression of tumor necrosis factor (TNF)-\(\alpha\) from infiltrating monocytes/macrophages and accelerated the proliferation of pericytes, which thereby increased the scar formation and subsequently exacerbated the functional recovery after SCI. Our findings suggest that POSTN is a critical extracellular component that modulates the scar formation in response to traumatic CNS injury and that the inhibitory nature of POSTN could be a therapeutic option for SCI.

**Materials and Methods**

**Animals**

Adult 8-week-old female C57BL/6 wild-type (WT) mice (\(n = 170\)) and POSTN knockout (\(\text{Postn}^{-/-}\)) mice\textsuperscript{17,24} (\(n = 96\)) were used in this study. The background strain of \(\text{Postn}^{-/-}\) mice used in this study is C57BL/6. Even when the impact force is consistent, the severity of SCI and the physiological responses are variable, depending on spinal cord size.\textsuperscript{25} Because most of the \(\text{Postn}^{-/-}\) mice showed slightly smaller overall body weights than WT mice,\textsuperscript{15} the standardization of the body weight as well as spinal cord size is necessary to examine the physiological differences between WT mice and \(\text{Postn}^{-/-}\) mice accurately. To achieve such standardization, the spinal cord sizes of WT mice and \(\text{Postn}^{-/-}\) mice were matched by calorie regulation.\textsuperscript{26} No signs of anxiety or aggressive behavior because of the calorie regulation were observed.

**Surgical Procedures**

Mice were anesthetized via an i.p. injection of pentobarbital (75 mg/kg). After laminectomy at the ninth thoracic level, a contusion SCI (70 kdyn) was induced using a highly reproducible computer-controlled impactor,\textsuperscript{27} an Infinite Horizons Impactor (Precision Systems Instrumentation, Lexington, KY) (WT mice, \(n = 134\); \(\text{Postn}^{-/-}\) mice, \(n = 82\)). All surgical procedures and experimental manipulations were approved by the Committee of Ethics on Animal Experiment in Faculty of Medicine, Kyushu University (Fukuoka, Japan). Experiments were conducted in accordance with the institutional guidelines and regulations for animal experiments.

**RNA Sequencing**

The sample preparation and data analysis were performed as described previously.\textsuperscript{28} Briefly, the mRNA-sequencing library was prepared using the NEBNext Ultra Directional Library Prep Kit for Illumina (San Diego, CA), and the samples were sequenced on an Illumina HiSeq-1500 system. The gene expression levels (fragments per kilobase of exon per million mapped sequence reads) were calculated using the TopHat version 2.0.11 and Cufflinks version 2.1.1 software programs (University of Maryland, College Park, MD) with default parameters. mRNA-sequencing data were deposited with accession codes DRA004534 (DNA Data Bank of Japan, Mishima, Japan, \url{http://trace.ddbj.nig.ac.jp/DRASeach}, last accessed January 10, 2017). An enrichment analysis for cellular components was performed based on the gene ontology database annotations with DAVID version 6.8 (Leidos Biomedical Research, Inc., Frederick, MD, \url{https://david.ncifcrf.gov}, last accessed March 4, 2016). Gene set enrichment analyses were performed using Gene Set Enrichment Analysis software program version 2.2.0 (Broad Institute, Cambridge, MA, \url{http://software.broadinstitute.org/gsea/index.jsp}, last accessed March 11, 2016) with a t-test metric for gene ranking and 1000 data permutations.

**Quantitative RT-PCR**

Total RNA was isolated from the spinal cord (\(n = 8\) in each time point and each group) using an RNeasy Micro Kit (Qiagen, Hilden, Germany). For cDNA synthesis, the reverse transcriptase reaction was performed using a Prime Script first-strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan). Quantitative RT-PCR was performed using primers specific to the genes of interest (Table 1) and SYBR Premix Dimmer Eraser (Takara Bio). The data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Histopathological Examination**

After the mice were transcardially fixed with 4% paraformaldehyde, the spinal cord was removed, dehydrated, and embedded in OCT compound. The frozen tissues were cut along the sagittal plane into sections (16 \(\mu\)m thick). For immunostaining, the sections were stained with primary antibodies against platelet-derived growth factor receptor \(\beta\) (PDGFR\(\beta\); pericyte marker, 1:200; rabbit; Abcam, Cambridge, UK), PDGFR\(\beta\) (1:100; rat; Biologend, San Diego, CA), PDGFR\(\beta\) (1:100; goat; R&D Systems, Minneapolis, MN), CD68 (monocyte/macrophage marker, 1:200; rat; Serotec, Oxford, UK), glial fibrillary acidic protein (GFAP), and nestin (neuroblastic marker, 1:500; mouse, 1:100; goat; Santa Cruz, Santa Cruz, CA). Immunostained sections were visualized using fluorescence microscopy (Axioplan 2 Imaging; Carl Zeiss, Jena, Germany).

**Data and Figure Ethics**

All data are presented as mean \(\pm\) standard error of the mean (SEM). The data were analyzed using the Mann–Whitney U test, one-way analysis of variance with a post hoc Bonferroni’s test, or Student’s t-test, as appropriate. The significance level was set at \(p < 0.05\).
Table 1  Primers Used for Quantitative RT-PCR

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*Accession numbers are for the NCBI Gene database: https://www.ncbi.nlm.nih.gov/gene

protein (astrocyte marker, 1:1000, rabbit; Dako, Glostrup, Denmark), glial fibrillary acidic protein (1:1000, rat; Life Technologies, Carlsbad, CA), Hu (neuronal marker, 1:1000, human; a gift from Dr. Robert Darnell, The Rockefeller University, New York, NY), perioxidin (1:200, goat (R&D Systems); 1:100, rat (R&D Systems); and 1:100, rabbit (BioVendor, Brno, Czech Republic), 5-bromodeoxyuridine (BrdU; 1:200, rat; Abcam), glutathione S-transferase [1:200, goat (R&D Systems); 1:100, rat (R&D Systems); and 1:100, rabbit (BioVendor, Brno, Czech Republic)], 5-bromodeoxyuridine (BrdU; 1:200, rat; Abcam), glutathione S-transferase—π (1:200, mouse; BD Biosciences, San Jose, CA), integrin αv (1:200, rabbit; Abcam), GAP43 (1:200, rabbit; Novus Biologicals, Littleton, CO), 5-hydroxytriptamine (1:200, goat; ImmunoStar, Hudson, WI), and Col1a2 (1:100, rabbit; Santa Cruz Biotechnology, Dallas, TX). Then, the sections were incubated with Alexa Fluor—conjugated secondary antibodies (1:200; Invitrogen, Carlsbad, CA). Nuclear counterstaining was performed using Hoechst 33342 (Molecular Probes, Eugene, OR). To evaluate the proliferating cells in the lesion areas, mice were injected with BrdU (100 mg/kg body weight) daily after SCI for the histopathological analysis. For BrdU detection, sections were pretreated with 2N HCl at 37°C for 15 minutes.

Image Acquisition and Quantitative Analysis

All images were obtained using a BZ-9000 digital microscope system (Keyence, Osaka, Japan). The area of the spinal cord was measured using the hematoxylin and eosin—stained axial section (n = 6 mice per group). For quantification of the area of scar tissue, the cross sections were stained with picrosirius red (n = 6 mice per group). For the quantification of proliferating pericytes in the injured spinal cords, we performed immunostaining with an anti-BrdU antibody and an anti-PDGFRβ antibody in each serial section at 150-μm intervals from side to side and captured 120 regions with ×100 magnification in each section to reconstruct a 2-mm section in each mouse (n = 6 mice per group). The algorithms for counting the number of proliferating cells were provided by the Dynamic cell count BZ-H1C measurement software program (Keyence), which selectively counted immunopositive particles in sizes ranging from 5 to 15 μm in both the x- and y-dimensions and automatically eliminated spurious particles. The algorithms for counting the number of serotonergic boutons were performed as described previously. We obtained images of ventral horn motor neurons at 5 mm distal to the lesion at ×1000 magnification of the sagittal section of each mouse for the reconstruction (n = 6 mice per group). To obtain one image of a motor neuron, 40 optical slices at 0.4-μm intervals were taken and projected into a single image. 5-HT—positive serotonergic boutons in each motor neuron were counted from that single optical slice image. We obtained images of 10 different motor neurons from the sagittal section of each mouse, for a total of 60 images of motor neurons across six mice per group. The total number of serotonergic boutons of each motor neuron per group was counted and presented in a boxplot.

Behavioral Analysis

The motor functions were evaluated with the locomotor open-field rating scale on the Basso Mouse Scale, which is specialized for the mouse hind limb motor function (n = 10 to 12 mice in each group). A team of two experienced examiners (T.S. and M.H.) evaluated each animal for 4 minutes and assigned an operationally defined score for each hind limb. Each mouse was assessed on postoperative days 1, 4, and 7 and weekly thereafter for 6 weeks. For the footprint analysis, the forelimbs and hind limbs of the mice were dipped in red and green dyes, respectively. A narrow runway (80 cm long and 4 cm wide) was lined with white paper as the animal walked across. The stride length was defined as the distance from the start to the end of a step with the back paw. The stride width was defined as the distance from the left outermost toe to the right outermost toe. Paw rotation was defined as the angle between the axis of the back paw and the midline axis of the body. All measurements were taken on each side for three consecutive steps and averaged (n = 8 mice per group). For the grip walk test, a grid walk was reconstructed for mice using two parallel pieces of wood. After 3 days of training, each mouse was allowed to cross the grip walk three consecutive times, and the number of grips was counted. A grip was defined as placing the toes on the rung while pushing off from the hind limb to move to the next step. The mice were evaluated using 50 cm of the grid with three difficulty levels: easy (50 steps, 1 cm apart), medium (every third step removed), and hard (every other step removed). The sum of
the number of grips for all three patterns was calculated in the grip walk analysis (n = 8 mice per group).

**Primary Culture of Pericytes**

Primary culture of mouse spinal cord pericytes was prepared as previously described. Briefly, spinal cords were removed from 8-week-old female C57BL/6 mice, minced, dissociated for 1 hour in papain and DNase I, and centrifuged through 22% bovine serum albumin to remove myelin. The resulting cells were cultured in endothelial cell growth media, consisting of Ham F12 supplemented with 10% fetal bovine serum, heparin, ascorbic acid, l-glutamine, penicillin/streptomycin, and endothelial cell growth supplement, on 6-well plates coated with type I collagen. The pericyte cultures were grown in endothelial cell growth media, with the medium changed every 3 days. On reaching confluence, cultures were harvested with trypsin and passaged. During the first two passages, pericyte cultures were grown in endothelial cell growth media, but on the third passage, they were switched to pericyte medium (ScienCell Research Laboratory, Carlsbad, CA). Previous studies have shown that the cultures of pericytes become highly purified after the third passage, as determined by the expression of the pericyte marker PDGFRβ. All functional assays were performed in serum-free Dulbecco’s modified Eagle’s medium.

**BrdU Incorporation Assay**

To investigate the influence of cytokines on pericyte behavior, pericytes were cultured in the presence of TNF-α (R&D Systems). The proliferation of pericytes was examined by adding 50 μg/mL BrdU to the culture medium and incubating for 24 hours. After fixation, samples were pretreated with 2N HCl at 37°C for 5 minutes. An anti-BrdU antibody and an anti-PDGFRβ antibody were used to identify proliferating pericytes. Bound antibodies were visualized using secondary antibodies with Alexa Fluor fluorescent conjugates.

**Flow Cytometry**

Spinal cord samples were prepared for flow cytometry, as previously described (n = 6 mice per group). Spinal cord samples were dissected and mechanically dissociated with collagenase (175 U/mL; Invitrogen) for 30 minutes at 37°C. The resulting suspension was pelleted by centrifugation and washed twice in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The cells were passed through a 40-μm nylon cell strainer (BD Biosciences) to isolate tissue debris from the cell suspension. The resulting suspension from the spinal cord was centrifuged, and the pellet was resuspended and incubated for 30 minutes on ice with the fluorescent antibodies. The samples were stained with anti-CD45, anti-CD11b, and anti−Gr-1 purchased from BioLegend. Before the analysis, propidium iodide was added to determine the cell viability. The samples were analyzed on a FACS Aria II flow cytometer (BD Biosciences). The data were analyzed using the FACS Diva software program version 6.1.3 (BD Biosciences).

**Chemotaxis Assays**

Transwell assays assessing cell migration or invasion potential were performed, as described previously. Briefly, macrophages obtained from mice were labeled with phosphatidylethanolamine-conjugated F4/80 antibody, and F4/80-positive cells were isolated using FACSAria II. Transwell assays were performed on 24-well plates with inserts (Corning Costar, Cambridge, MA), in accordance with the manufacturer’s instruction. A total of 5 × 10⁴ macrophages were cultured in the upper chamber and allowed to migrate or invade for 12 to 18 hours before fixation for Diff-Quick staining (Sysmex, Kobe, Japan). Recombinant mouse POSTN and anti-POSTN antibody were purchased from R&D Systems.

**Bone Marrow Chimeras**

Bone marrow chimeras were prepared as previously described. In brief, chimeras were prepared by subjecting sex-matched recipient WT mice or Postn−/− mice (n = 8 mice per group) to lethal whole-body irradiation (10 Gy). The recipient mice were then reconstituted with 1 × 10⁷ bone marrow cells derived from cytomegalovirus enhancer and chicken β-actin promoter—enhanced green fluorescence protein (GFP) transgenic mice. Chimeric mice were used 8 weeks after bone marrow transplantation.

**POSTN Monoclonal Antibody Production**

Hybridoma cell lines secreting mouse monoclonal antibody against POSTN were purchased from ATCC (Manassas, VA). For antibody purification, hybridoma cells were grown in culture medium and the supernatants were harvested. The culture medium consisted of hybridoma—serum free media (Invitrogen) supplemented with IL-6 (1 ng/mL), 10% fetal bovine serum, l-glutamine, and penicillin/streptomycin. The mouse POSTN monoclonal antibodies were purified by protein G affinity chromatography. The protein concentrations of the obtained antibodies were measured by the NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The purified antibody blocked the monocytic/macrophage migration, as confirmed by the Transwell assays, and was then diluted in saline and injected into mice after SCI at a dose of 1 mg/kg body weight.

**Statistical Analysis**

The Wilcoxon rank sum test was used to compare the median values of the following between WT mice and Postn−/− mice: number of BrdU-positive/PDGFRβ-positive cells in injured...
spinal cords; picrosirius red—positive area; GAP43-positive area; 5-HT—positive boutons; the expression levels of Col1a1, Col1a2, Col3a1, Col4a1, TNF-α, PDGF-B, and vascular endothelial growth factor in spinal cords; the area of naive spinal cords; the number of BrdU-positive cells in naive spinal cords; the number of PDGFRβ-positive cells in naive spinal cords; the footprint scores; the grip walk scores; the number of infiltrating monocytes/macrophages; and the expression level of TNF-α in sorted monocytes/macrophages. For multiple comparisons of the differences in the number of proliferating pericytes with or without TNF-α exposure, the number of migrating macrophages with or without recombinant POSTN (rPOSTN) exposure, and the gene expression levels of TNF-α with or without rPOSTN exposure, a one-way factorial analysis of variance with a post hoc Tukey-Kramer test was performed. For multiple comparisons of the differences in the functional recovery of Basso Mouse Scale scores between the SCI groups over time, a two-way repeated-measures analysis of variance with a post hoc Tukey-Kramer test was performed. In all of the statistical analyses, significance was defined as $P < 0.05$. The values for groups are presented as the averages ± SEM. All statistical analyses were performed using the JMP software program version 11 (SAS Institute, Cary, NC).

**Results**

**Prominent Expression of POSTN after SCI**

To explore factors involved in scar formation, we performed a genome-wide screening for mRNAs expressed in an injured spinal cord after SCI. The heat map visually revealed dynamic changes in the expression of genes in an injured spinal cord compared to those in a naive spinal cord (Figure 1A). A gene ontology term analysis indicated that the genes involved in the extracellular region were particularly enriched (Figure 1B). In addition, a gene set enrichment analysis of the injured spinal cord showed significant up-regulation of the gene sets involved in the extracellular region compared with the naive spinal cord (Figure 1C and Supplemental Figure S1). In the extracellular region—related genes, a significant difference in the expression of POSTN was observed at 7 days after injury by Cuffdiff (Figure 1, D and E). A quantitative RT-PCR analysis showed that the expression of POSTN prominently increased at 7 days after injury, which remained detectable for 6 weeks (Figure 1F). Consistent with these findings for the POSTN expression, a significant increase in POSTN protein expression was confirmed by immunofluorescence analysis for 6 weeks after SCI (Figure 1G).

**Selective Expression of POSTN in Pericytes**

We next examined the type of cells expressing POSTN in the injured spinal cord. We hypothesized that POSTN-expressing cells would be monocytes/macrophages, astrocytes, neurons, and pericytes, because these cells constitute part of the lesion after SCI. No POSTN expression was detected in CD68-positive monocytes/macrophages, glial fibrillary acidic protein—positive astrocytes, or Hu-positive neurons; instead, POSTN was predominantly expressed in PDGFRβ-positive pericytes in the injured spinal cord (Figure 2, A—D, and Supplemental Figure S2).

**Proliferation of Pericytes and Scar Formation Were Decreased in POSTN Knockout Mice**

The elevated expression of POSTN in the injured spinal cord led us to analyze the effect of POSTN on the pathohistological phenotype and functional outcome after SCI. We first confirmed a lack of any significant differences in the size of spinal cords, the number of proliferating cells, and the number of pericytes between WT mice and POSTN knockout (Postn$^{-/-}$) mice before SCI (Supplemental Figure S3). We then investigated the pathohistological differences of the injured spinal cord between the WT mice and Postn$^{-/-}$ mice. Interestingly, we found that the area of PDGFRβ-positive pericytes at the lesion site in Postn$^{-/-}$ mice was significantly smaller than in WT mice (Figure 3A). Furthermore, the number of proliferating pericytes in the group of Postn$^{-/-}$ mice was significantly fewer than that in the group of WT mice at 2 weeks after SCI (Figure 3, A and B). In addition to the distribution of pericytes in the injured spinal cord, we evaluated the differences in the scar formation between the two groups of mice because the pericytes constitute a fraction of the scar. Picrosirius red staining, which is the specific staining for fibrillar collagen and reflects fibrous scar formation, showed a significantly smaller amount of collag enous scar in Postn$^{-/-}$ mice than in WT mice (Figure 3, C and D). We also confirmed that the pericytes expressed collagen proteins after SCI (Figure 3E). The gene expression of collagen 1a1 and collagen 1a2, but not collagen 3a1 or collagen 4a1, was significantly lower in Postn$^{-/-}$ mice than in WT mice (Figure 3F). These results suggest that POSTN was involved in the proliferation of pericytes and in scar formation after SCI.

**POSTN Deficiency Ameliorates Functional Recovery after SCI**

We next compared the recovery of the locomotor function after SCI between WT mice and Postn$^{-/-}$ mice. The Basso Mouse Scale score, the footprint analysis, and the grip walk test revealed significantly better functional recovery in Postn$^{-/-}$ mice at 6 weeks after SCI compared with the WT mice (Figure 4, A—C). We further evaluated the axonal regeneration in the injured spinal cord between the WT mice and Postn$^{-/-}$ mice using immunofluorescence analyses. GAP43-positive regenerative fibers at the lesion site increased in Postn$^{-/-}$ mice compared to WT mice (Figure 4, D and E). In addition, a larger number of serotonergic 5-HT axons innervated the motor neurons in the ventral horn of the
lumbar spinal cord in Postn<sup>−/−</sup> mice than in WT mice (Figure 4, F and G). These findings suggest that POSTN was involved in the impairment of functional recovery after SCI and in the decreased axonal regeneration at the lesion site.

**POSTN Promotes TNF-Mediated Proliferation of Pericytes after SCI**

As previously reported, neurohumoral factors, such as tumor necrosis factor (TNF)-α, PDGF-B, and vascular endothelial growth factor, regulate the proliferation of pericytes in the CNS. To examine the factors involved in the proliferation of pericytes after SCI in WT mice and Postn<sup>−/−</sup> mice, we examined the gene expression of these neurohumoral factors in both groups of mice. The gene expression of TNF-α was significantly lower in Postn<sup>−/−</sup> mice than in WT mice at 7 and 14 days after SCI (Figure 5A). In contrast, the gene expressions of PDGF-B and vascular endothelial growth factor were comparable in both groups of mice (Figure 5A).

We then examined the direct effect of TNF-α on the proliferation of pericytes isolated from an adult spinal cord. The number of proliferating pericytes after exposure to TNF-α was significantly higher than that without TNF-α exposure (Figure 5B). The number of proliferating pericytes increased significantly with an increase in TNF-α, indicating that TNF-α promotes the proliferation of pericytes in a dose-dependent manner (Figure 5C).

**POSTN Regulates the Migration of Monocytes/Macrophages into the Injured Spinal Cord**

Previous studies have suggested that POSTN modulates the secretion of inflammatory cytokines and chemokines through integrin receptors, such as αvβ1, αvβ3, and αvβ5. In addition, in injured spinal cords, infiltrating monocytes/macrophages are major sources of cytokines and chemokines. We found that the integrin αv was expressed in monocytes/macrophages at the lesion site, whereas this integrin was rarely

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**Figure 1** A time-course mRNA screening reveals prominent expression of POSTN in the injured spinal cord. A: A heat map shows differential gene expression in the naive and injured spinal cord at 7 days after SCI. B: A gene ontology (GO) term analysis of overexpressed (>50-fold change) genes in the mRNA-sequencing analysis of the injured spinal cord compared to those of the naive spinal cord. Lists show the top seven GO terms obtained ranked by P value (Fisher’s exact test with the Benjamini-Hochberg correction). C: Gene set enrichment analysis plots show up-regulation of extracellular region–related genes in the injured spinal cord at 7 days after SCI. D: A volcano plot of gene expression differences between the naive spinal cord and the injured spinal cord. Red dots indicate significantly up-regulated or down-regulated genes. E: Wiggle plots showing the coverage of each exon for POSTN before injury and after injury. F: The significantly increased mRNA expression of POSTN analyzed via quantitative RT-PCR of spinal cord before and after SCI. G: An immunohistochemical analysis of the injured spinal cord stained with POSTN (green). The asterisk shows the lesion epicenter. The dotted line indicates the outline of the injured spinal cord. Data are presented as means ± SEM (F). n = 8 mice at each time point (F). Scale bar = 500 μm (G).
expressed in oligodendrocytes, neurons, astrocytes, or pericytes (Figure 6A). These findings suggest that POSTN would have little direct effect on pericytes but have some effect on monocytes/macrophages. Because POSTN was reported to regulate cell migration through the integrin receptors,41 we focused on the effect of POSTN on the migratory potential of monocytes/macrophages. We performed a series of migration and invasion assays to examine the capacity of POSTN to attract monocytes/macrophages (Figure 6B). Conditioned media with rPOSTN attracted significantly more monocytes/macrophages than those without rPOSTN (Figure 6C). In addition, the migration toward POSTN was significantly enhanced in an rPOSTN dose-dependent manner (Figure 6D).

To further address whether an anti-POSTN antibody could attenuate the promoting effect of monocyte/macrophage migration, we examined the capacity of POSTN to attract monocytes/macrophages with or without the anti-POSTN antibody. Conditioned media with both rPOSTN and anti-POSTN antibody attracted a smaller number of monocytes/macrophages than media conditioned with both rPOSTN and isotype control IgG (Figure 6, E and F). These results indicate that POSTN exhibits a potent capacity to attract monocytes/macrophages.

We quantified the number of monocytes/macrophages infiltrating into the injured spinal cords of WT and Postn−/− mice in vivo using flow cytometry (Figure 6G). Consistent with the response to rPOSTN exposure in monocytes/macrophages in vitro (Figure 6, C–F), we observed smaller numbers of infiltrating monocytes/macrophages in Postn−/− mice than in WT mice at 7 days after injury (Figure 6H). We also found that the expression of TNF-α in monocytes/macrophages sorted from Postn−/− mice was significantly lower than that derived from WT mice at 7 days after injury (Figure 6I). We then examined the direct effect of POSTN on the TNF-α expression derived from monocytes/macrophages in vitro.

Figure 2  POSTN expression was observed selectively in pericytes after SCI. A–D: An immunohistochemical analysis of the injured spinal cord stained with CD68 (monocyte/macrophage marker; A), glial fibrillary acidic protein (GFAP; astrocyte marker; B), Hu (neuronal marker; C), PDGFRβ (pericyte marker; D), or POSTN at 7 days after SCI is shown. Scale bar = 100 μm (A–D).
The gene expression of TNF-α was significantly up-regulated with rPOSTN exposure compared to that observed without rPOSTN exposure (Figure 6J). In contrast, the expression of TNF-α from the microglia/macrophages (BV2: microglial cell line) was unaffected by exposure to rPOSTN (Supplemental Figure S4). These results together suggest that POSTN has direct effects on monocytes/macrophages for promoting the migratory potential and up-regulating the expression of TNF-α.

To confirm the effect of POSTN in promoting the infiltration of monocytes/macrophages histologically, we generated bone marrow chimeric mice by transplanting bone marrow cells from mice that ubiquitously expressed GFP into lethally irradiated WT or Postn−/− mice (Supplemental Figure S5A). In both Postn−/−-GFP mice and WT-GFP mice, GFP/CD68-positive monocytes/macrophages exhibited a wild-type phenotype. These chimeric mice enable us to examine whether the absence of POSTN affects the number of infiltrating blood-derived monocytes/macrophages in vivo. The immunofluorescence staining of infiltrating monocytes/macrophages with antibodies against GFP, CD68, and POSTN revealed a smaller number of infiltrating monocytes/macrophages in Postn−/−-GFP chimeric mice than in WT-GFP chimeric mice (Supplemental Figure S5B).

Figure 3  The proliferation of pericytes and scar formation are decreased in POSTN knockout mice. A and B: The results of a comparison of the number of PDGFRβ-positive/5-bromodeoxyuridine (BrdU)-positive cells in WT and Postn−/− mice at 2 weeks after SCI is shown. The asterisk indicates the lesion epicenter. The dotted line indicates the outline of the injured spinal cord. The three right panels are magnifications of the boxed areas in the left panels. C and D: Picrosirius red staining showed significantly less scar formation in Postn−/− mice than in WT mice at 2 weeks after SCI. The asterisk indicates the lesion epicenter. E: An immunohistochemical analysis of the injured spinal cord stained with PDGFRβ (green), Col1a2 (red), and Hoechst (blue) is shown. F: The gene expressions of collagens (col1a1, col1a2, col3a1, and col4a1) in the injured spinal cord are shown. Data are presented as means ± SEM (B, D, and F). n = 6 mice per group (A–D); n = 8 mice at each time point (F). *P < 0.05, Wilcoxon rank sum test. Scale bars: 500 μm (A, left panels, and C); 100 μm (A, right three panels); 50 μm (E).
Administration of POSTN-Neutralizing Antibody Ameliorates Functional Recovery after SCI

We compared the locomotor function with or without administration of an antagonistic monoclonal antibody targeting POSTN (POSTN-mAb) into WT mice after SCI. In the POSTN-mAb group, the mice received daily injections of the POSTN-mAb from 4 days to 2 weeks after injury, and in the control IgG group, the mice received daily injections of an isotype control IgG antibody from 4 days to 2 weeks after injury (Figure 7A). Significantly better functional recovery after SCI was observed in the POSTN-mAb group compared to the control IgG group from 2 to 6 weeks after SCI (Figure 7, B–D). In addition, the scar formation was significantly smaller in the POSTN-mAb group than in the control IgG group (Figure 7, E and F). We then investigated whether administering POSTN-mAb even in the early-chronic phase (from 2 weeks onward in mice) could promote functional recovery after SCI, because the expression of POSTN was consistently observed from 2 to 6 weeks after injury (Figure 1F). We treated WT mice with the POSTN-mAb or with the control IgG from 2 to 4 weeks after SCI (Supplemental Figure S6A); however, the functional recovery was comparable between the POSTN-mAb group and the control IgG group (Supplemental Figure S6B). These results indicate that there is a therapeutic time window in which administering the POSTN-mAb can improve the functional outcome after SCI.

Discussion

In this study, we demonstrated three significant findings. First, we showed that POSTN was involved in scar formation and in the proliferation of pericytes after SCI. Second, we found that POSTN promoted the infiltration capacity of
monocytes/macrophages and their expression of TNF-α, which increased the proliferation of pericytes. Third, we succeeded in improving the functional recovery through the reduction of scar formation by inhibiting POSTN after SCI. Overall, we demonstrated that neutralizing POSTN could attenuate the regeneration-suppressive environment at the site of injury, providing useful information for establishing a therapeutic strategy for CNS injury.

POSTN has been known to modulate biological immune responses, such as producing type 2 helper T-cell allergic reaction and facilitating tumor growth acceleration or tumor metastasis. In this study, POSTN facilitated the infiltration of monocytes/macrophages into the injured spinal cord and promoted the expression of TNF-α derived from the infiltrating monocytes/macrophages. We also confirmed that TNF-α promotes the proliferation of pericytes. Thus, the suppressed proliferation of pericytes after SCI in Postn-/- mice was attributed to the reduced expression of TNF-α in the injured spinal cord (Figure 5). Under these conditions, the proliferation of pericytes after SCI would be indirectly affected by POSTN through TNF-α derived from infiltrating monocytes/macrophages. Herein, we propose that scar formation regulated by POSTN is linked to the functional outcome after SCI (Figure 7G).

Regarding the mechanism by which POSTN enhances the infiltration of monocytes/macrophages into the injured spinal cord, focal adhesion kinase (FAK)/Akt signaling may be involved. Integrin αvβ3 receptor, which is the POSTN binding receptor in monocytes/macrophages, is known to mediate the phosphorylation of FAK. In the peripheral circulatory system, the migration potential of monocytes/macrophages is enhanced through the up-regulation of the FAK/Akt. Even after CNS injury, infiltrating monocytes/macrophages have been reported to exhibit chemotactic responses via phosphorylation of FAK. These findings suggest that the POSTN-integrin axis is associated with the migration potential of monocytes/macrophages through the FAK/Akt pathway in the injured spinal cord. In addition, during the process of ischemic heart remodeling, the activation of FAK signaling in macrophages was found to promote the scar formation, supporting the notion that the POSTN-integrin axis in monocytes/macrophages is also related to the scar formation after the CNS injury.

We herein found that POSTN could promote the expression of TNF-α in monocytes/macrophages rather than microglia/macrophages in vitro. However, it is extremely difficult to selectively evaluate the direct effects of POSTN on the activation of microglia/macrophages.

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**Figure 5** The expression of tumor necrosis factor (TNF)-α is decreased in POSTN knockout mice, and TNF-α regulates the proliferation of pericytes. A: The gene expressions of TNF-α, PDGF-B, and vascular endothelial growth factor (VEGF) in the injured spinal cord are shown. B: An immunohistochemical analysis of isolated pericytes stained with 5-bromodeoxyuridine (BrdU; green), PDGFRβ (red), and Hoechst (blue). C: The ratio of the number of BrdU-positive proliferating pericytes/total number of pericytes after exposure to different concentrations of recombinant TNF-α in proliferation assays. Data are presented as means ± SEM (A and C). n = 8 mice at each time point (A); n = 5 per group (C). *P < 0.05, Wilcoxon rank sum test (A) or analysis of variance with the Tukey-Kramer post hoc test (C). Scale bar: 100 μm (B).
POSTN Mediates Scar Formation after SCI

**Figure 6** POSTN promotes the infiltration of monocytes/macrophages into the injured spinal cord and enhances the expression of tumor necrosis factor (TNF)-α from monocytes/macrophages. **A**: An immunohistochemical analysis of the injured spinal cord stained with integrin αv (red) and respective indicated markers (green). **B**: Experiment summary schematic of Transwell assays. **C**: Representative images of isolated monocytes/macrophages that migrated toward different concentrations of recombinant POSTN (rPOSTN) in Transwell assays. **D**: The results of a comparison of the number of migrating macrophages toward conditioned medium with or without rPOSTN. **E**: Representative images of isolated monocytes/macrophages that migrated toward conditioned medium with or without rPOSTN, and with monoclonal POSTN antibody or control IgG in Transwell assays. **F**: The results of a comparison of the number of monocytes/macrophages migrating toward conditioned medium with or without rPOSTN, and with monoclonal POSTN antibody or control IgG. **G**: A flow cytometry analysis shows the fractions of CD45<sup>hi</sup>/CD11b<sup>hi</sup> monocytes/macrophages infiltrating into injured spinal cords at 7 days after SCI in WT and Postn<sup>−/−</sup> mice. **H**: The gene expression of TNF-α in the monocytes/macrophages after exposure to different concentrations of rPOSTN. Data are presented as means ± SEM (D, F, H–J), n = 6 per group (D, F, H–J). *P < 0.05, analysis of variance with the Tukey-Kramer post hoc test (D, F, and J) or Wilcoxon rank sum test (H and I). Scale bars: 100 μm (C and E); 50 μm (A). GFAP, glial fibrillary acidic protein; GST, glutathione S-transferase.

In *vivo*, because both the infiltration of monocytes/macrophages and the cytokine secretion capacity of monocytes/macrophages are intimately linked to the activation of microglia/macrophages. The interaction between the activation of monocytes/macrophages and the activation of microglia/macrophages is a potential limitation in evaluating the cellular-selective activation that occurs after POSTN exposure. Further investigation is necessary to evaluate the activation of microglia/macrophages using a mouse model with the conditional ablation of microglia/macrophages after SCI.

Although pericytes have been confirmed to be a source of scar tissue, few studies have investigated the role of pericytes in scar formation after CNS injury. In this study, we found that the pericytes proliferated at the lesion site and expressed type I collagen. As previously described,
pericytes do produce type I collagen rather than other types of collagen.\textsuperscript{55} In addition, in Postn\textsuperscript{-/-} mice, type I collagen production is down-regulated in the tracheal epithelial cells as well as in infarcted heart tissue,\textsuperscript{24,56} indicating the relationship between POSTN secretion and collagen production. The accumulation of collagen has been reported to prevent CNS regeneration, such as axonal sprouting and remyelination,\textsuperscript{57} suggesting that an increased number of pericytes inhibited axonal regeneration and exacerbated the functional recovery after SCI.

The extracellular matrix has been reported to be involved in the functional outcome after the CNS injury.\textsuperscript{58} We showed that POSTN in the scar impaired the functional recovery after SCI. Chondroitin sulfate proteoglycans, which also comprise the extracellular part of the injured CNS, are a possible target for the treatment of SCI.\textsuperscript{7} In a previous study, the administration of the chondroitinase ABC dissolved the accumulated chondroitin sulfate proteoglycans at the lesion site and promoted axonal regeneration, resulting in a better functional outcome after SCI.\textsuperscript{59} However, for establishing a treatment protocol, further studies are necessary.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure7.png}
\caption{Inhibiting POSTN ameliorates functional recovery and decreases scar formation after SCI. A: A schematic illustration of the antibody injection protocols for neutralizing POSTN after SCI. Mice were given an antagonistic monoclonal antibody targeting POSTN (POSTN-mAb) or a control IgG i.p. with single daily dosing from 4 days to 2 weeks after SCI. B: The time course of the functional recovery based on the Basso Mouse Scale score in the POSTN-mAb and the control IgG subacute treatment groups is shown. C and D: The results of the footprint analyses and grip walk test of POSTN-mAb and the control IgG subacute treatment groups at 6 weeks after SCI are shown. E and F: Picrosirius red staining revealed significantly less scar formation in the POSTN-mAb group than in the control IgG group at 2 weeks after SCI. G: A schematic illustration of the role of POSTN in scar formation of an injured spinal cord brought about by pericytes and monocytes/macrophages. \( n = 10 \) mice per group (B); \( n = 8 \) per group (D); \( n = 6 \) mice per group (E and F). *\( P < 0.05 \), two-way repeated-measures analysis of variance with the Tukey-Kramer post hoc test (B) or Wilcoxon rank sum test (D and F). Scale bar = 500 \( \mu \text{m} \). KO, knockout; TNF-\( \alpha \), tumor necrosis factor-\( \alpha \).}
\end{figure}
for human SCI, chondroitinase ABC administration carries a risk of infection with microorganisms, because chondroitinase ABC is extracted from microbial proteolytic product.\textsuperscript{60} Given the risk associated with that procedure, the administration of neutralizing antibody against POSTN may be a safer, more promising therapeutic option for SCI, by developing a chimeric or a humanized POSTN antibody.

Previous reports have shown that the suppression of TNF-\(\alpha\) attenuated tissue damage and promoted functional recovery after SCI.\textsuperscript{61,62} However, the mechanism by which this TNF-\(\alpha\) suppression leads to such histological and functional improvement is unclear. In the present study, suppressing TNF-\(\alpha\) secretion in Postn\textsuperscript{-/-} mice reduced the proliferation of pericytes, resulting in a reduction in the scar formation with better axonal regeneration at the lesion site. Although substantial research has focused on the establishment of therapeutic approaches for SCI by modulating proinflammatory reactions during the acute phase (within 4 days after injury),\textsuperscript{63} our findings suggest that modulating such immune reactions during the subacute phase (from 4 days after injury onward) also could improve the functional outcome after SCI.

The direct blockade of secreted proinflammatory cytokines, such as TNF-\(\alpha\), IL-6, and IL-1\(\beta\), successfully improved the functional outcome after CNS injury, as previously described.\textsuperscript{65–68} However, such therapeutic approaches of modulating immune responses may unexpectedly impair the human circulatory system and result in the malfunction of immune organs. Indeed, in clinical practice, some patients treated with TNF-\(\alpha\) antibody or IL-6 antibody have experienced severe adverse effects, such as parasitic disease, active tuberculosis, demyelinating disorder, and pancytopenic syndrome.\textsuperscript{65,66} The expression of POSTN is usually observed in the developmental stage, with hardly any endogenous POSTN expression in the adult organs\textsuperscript{19}; therefore, the administration of neutralizing POSTN antibody for SCI patients is expected to cause few adverse effects. A therapeutic option targeting POSTN would pave the way for establishing a treatment regimen for CNS injury.

In conclusion, we clarified that POSTN regulates interactive scar tissue formation by pericytes and monocytes/macrophages. Inhibiting POSTN reduced the scar formation as well as promoted functional recovery after SCI, showing evidence that targeting POSTN is important for establishing treatment methods for CNS injury.

Acknowledgments

K.Y. designed and performed most of the experiments with technical help from K.Ko., T.S., M.H., and K.Ki.; Y.O., A.H., K.O., K.I., S.Y., A.K., and Y.I. provided experimental support and ideas for the project; and S.O. designed the studies, supervised the overall project, and performed the final manuscript preparation.

**Supplemental Data**

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2016.11.010.

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