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Acupuncture-mediated inhibition of inflammation facilitates significant functional recovery after spinal cord injury

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ABSTRACT

Here, we first demonstrated the neuroprotective effect of acupuncture after SCI. Acupuncture applied at two specific acupoints, Shuigou (GV26) and Yanglingquan (GB34) significantly alleviated apoptotic cell death of neurons and oligodendrocytes, thereby leading to improved functional recovery after SCI. Acupuncture also inhibited caspase-3 activation and reduced the size of lesion cavity and extent of loss of axons. We also found that the activation of both p38 mitogen-activated protein kinase and resident microglia after injury are significantly attenuated by acupuncture. In addition, acupuncture significantly reduced the expression or activation of pro-nerve growth factor, proinflammatory factors such as tumor necrosis factor-α, interleukin-1β, interleukin-6, nitric oxide synthase, cycloxygenase-2, and matrix metalloprotease-9 after SCI. Thus, our results suggest that the neuroprotection by acupuncture may be partly mediated via inhibition of inflammation and microglial activation after SCI and acupuncture can be used as a potential therapeutic tool for treating acute spinal injury in human.

Introduction

Spinal cord injury (SCI) induces massive apoptotic death of neurons in the gray matter (GM) and oligodendrocytes in the white matter (WM), resulting in demyelination, and consequently, spinal cord dysfunction (Casha et al., 2001; Liu et al., 1997; Springer et al., 1999). Inflammation is one of the major secondary degenerative responses that exacerbates the pathogenesis of SCI by inducing neuronal and oligodendroglial apoptosis (Bao and Liu, 2004; Bareyre and Schwab, 2003). Microglia, endogenous immune cells in the central nervous system (CNS), play a pivotal role in inducing inflammatory responses by expressing inflammatory mediators, such as proinflammatory cytokines, cycloxygenase (COX)-2, inducible nitric oxide synthase (iNOS) (Block and Hong, 2005), pro-nerve growth factor (proNGF) (Yune et al., 2007) and reactive oxygen species (ROS), upon activation by several inflammatory stimulators (Min et al., 2003, 2004; Qin et al., 2004), and thereby contribute to neurodegenerative processes after injury. Thus, the development of therapeutic drugs that effectively prevent microglial activation after SCI is crucial.

Acupuncture has long been employed as a treatment for numerous diseases in Oriental medicine. Several therapeutic effects of acupuncture have been known, including analgesia, pain control, promotion of homeostasis and improvement in brain blood circulation (Kim et al., 2005; Uchida et al., 2000). Also, acupuncture has been used to relieve neurological dysfunction in neurodegenerative disorders, such as Parkinson's disease (PD) and ischemia (Chuang et al., 2007; Jeon et al., 2008; Park et al., 2003). In addition, a number of preclinical and clinical studies demonstrate that manual acupuncture or electro-acupuncture (EA) induces functional improvement in motor function in CNS injuries, including stroke and SCI (Kang, 2007; Takakura et al., 1996; Wang et al., 2003). Furthermore, several reports show that acupuncture or EA effectively attenuates ischemia-induced cerebral infarction and apoptosis (Guo et al., 2002; Jang et al., 2003; Si et al., 1998; Yang et al., 1999). In particular, a recent report shows that acupuncture inhibits microglial activation and inflammatory reactions in a mouse model of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD (Kang et al., 2007).

Given that there are data suggesting the neuroprotective effects of acupuncture in neurodegenerative diseases and CNS injuries, we hypothesized that acupuncture would ameliorate functional outcome after SCI. Here, we report that acupuncture treatment inhibits

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apoptotic cell death of neurons and oligodendrocytes by inhibiting microglial activation and thereby improves functional recovery after injury. Therefore, our data suggest that acupuncture can be applied as a therapeutic tool for improving locomotor function in spinal cord injured patients.

Materials and methods

Spinal cord injury

Adult rats [Sprague–Dawley, male, 250–300 g; Sam:TacN (SD) BR; Samtako, Osan, Korea] were anesthetized with chloral hydrate (500 mg/kg), and a laminectomy was performed at the T9-T10 level, exposing the cord beneath without disrupting the dura. The spinous processes of T8 and T11 were then clamped to stabilize the spine, and the exposed dorsal surface of the cord was subjected to contusion injury (10 g × 25 mm) using a New York University impactor as described previously (Yune et al., 2007). For the sham-operated controls, the animals underwent a T10 laminectomy without weight-drop injury. All surgical interventions and postoperative animal care were performed in accordance with the Guidelines and Polices for Rodent Survival Surgery provided by the Animal Care Committee of the Kyung Hee University.

Acupuncture application and selection of neuroprotective acupoint after injury

Acupuncture was applied at each specific acupoint (Yin et al., 2008) immediately after injury. Under still anesthesia after injury, stainless-steel needles of 0.20 mm in diameter were inserted to a depth of 4–6 mm at each acupoint bilaterally, turned at a rate of two spins per second for 30 s. The needles were retained for 30 min. Then, acupuncture was applied to rats once a day for 2 weeks without anesthesia using an immobilization apparatus designed by our laboratory (Fig. 1B). We used rat received injury but not received any acupuncture treatment as a control. For another control experiment, a simulated acupuncture treatment with a toothpick at each acupoint was also performed as described (Cherkin et al., 2009). In brief, the skin of each specific acupoint was tapped with the tip of a toothpick to imitate an acupuncture needle insertion. The acupiont was then gently touched with the tip of a toothpick, and the toothpick was turned at a rate of two spins per second for 30 s. After 30 min, to simulate withdrawal of the needle, a toothpick momentarily touched the skin of the acupoint and was then quickly pulled away (Cherkin et al., 2009).

To find the most effective acupoint, we compared the effect of several selected acupoints having potential neuroprotective effects by counting the number of viable ventral motor neurons (VMN) after SCI. The acupoints applied were GV26 (Shuigou), GB34 (Yanglingquan), ST36 (Zusanli), BL60 (Kunlun), BL40 (Weizhong), GB39 (Xuanzhong) and SP6 (Sanyinjiao) (Yin et al., 2008), (Fig. 1A). Briefly, injured rats were divided into eight groups: control, GV26, GB34, ST36, BL60, BL40, GB39 and SP6 ($n = 5$/group). Rats received acupuncture treatment immediately after injury and then once a day. Four days after injury, rats were anesthetized and perfused, and cross frozen tissue sections (20 μm) were then cut. For quantification, serial transverse sections were collected every 500 μm from 2.5 mm rostral to 2.5 mm caudal to the lesion epicenter (total of 11 sections each animal) and stained with Cresyl violet, acetate and the number of viable VMN over 30 μm in diameter was counted as previously described (Yune et al., 2007, 2008). The cells were manually counted from each field using Metamorph software (Molecular devices, Sunnyvale, CA). As shown in Fig. 1C, GV26 and GB34 showed higher numbers of viable VMN than other acupoints after injury. Thus, we used the two specific acupoints, GV26 and GB34, throughout our experiments.

Tissue preparation

After SCI, animals were anesthetized with chloral hydrate and perfused via cardiac puncture initially with 0.1 M PBS (pH 7.4) and subsequently with 4% paraformaldehyde in 0.1 M phosphate buffer. A 20 mm section of the spinal cord, centered at the lesion site, was dissected out, post-fixed by immersion in the same fixative overnight and placed in 30% sucrose in 0.1 M PBS. The segment was embedded in OCT for frozen sections as described previously (Yune et al., 2007).

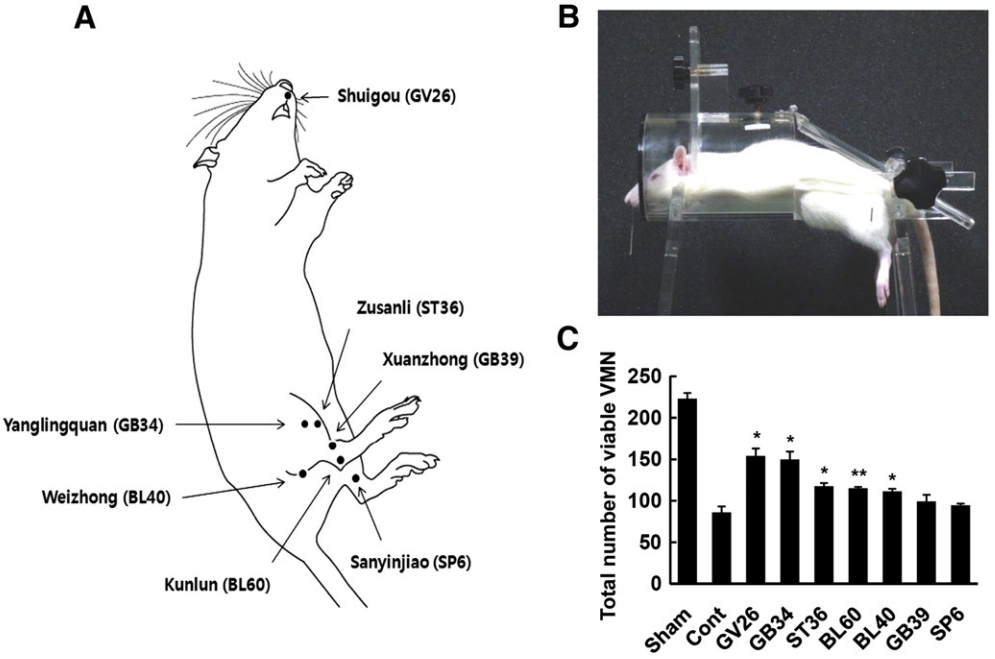


Fig. 1. Selection of neuroprotective acupoints after SCI. A. Locations of the acupoints tested. B. Photograph showing an immobilization apparatus for acupuncture treatment without anesthesia. C. Neuroprotective effect of each specific acupoint, examined by counting viable motor neurons in the ventral horn at 4 days after injury as described in the Methods section ($n = 5$/group). Data are presented as means ± SD from three separate experiments. *$p<0.05$; **$p<0.01$.

Longitudinal and cross frozen tissue sections were then cut at 10 or 20 μm on a cryostat (CM1850; Leica, Germany).

TUNEL and immunohistochemical staining

One or 5 days after injury, serial coronal or longitudinal spinal cord sections (10 μm thickness) were collected every 100 μm sections (total 40 sections for neurons and 100 sections for oligodendrocytes) and processed for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) and then for immunocytochemistry using specific cell type makers: NeuN (1:100, Milipore, Billerica, MA) for neurons; CC1 (1:100; Oncogene, Cambridge, MA) for oligodendrocytes. Only double-labeled cells were considered and counted as TUNEL-positive neurons (1 day) in the GM or oligodendrocytes (5 days) in the WM. For quantification, serial transverse sections (20 μm thick) were collected every 100 μm from 2 mm rostral to 2 mm caudal to the lesion epicenter for neurons (total 40 sections) or 5 mm rostral to 5 mm caudal for oligodendrocytes (total 100 sections), and double-positive neurons (NeuN + TUNEL) in the GM and oligodendrocytes (CC1 + TUNEL) in the WM counted. Some sections were processed for immunofluorescence with antibodies against phosphorylated p38 mitogen-activated protein kinase (p-p38MAPK; 1:100; Cell Signaling Technology, Danvers, MA), proNGF (1:1000; Alomone Labs, Jerusalem, Israel), OX-42 (1:100, Millipore), cleaved caspase-3 (1:100; Millipore) and CC1 (1:100). For double labeling, fluorescein isothiocyanate (FITC) or cyanine 3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used. Also, nuclei were labeled with DAPI according to the protocol of the manufacturer (Invitrogen, Carlsbad, CA). In controls, reaction to the substrate was absent if the primary antibody was omitted or if the primary antibody was replaced by a non-immune, control antibody. Some sections were also stained for histological analysis with Cresyl violet acetate. For quantification of cleaved caspase-3-positive oligodendrocytes (cleaved caspase-3/CC1 double positive), serial transverse sections (10 μm thickness) were collected every 100 μm sections from rostral and caudal 4 mm to the lesion site (total 80 sections). Cleaved caspase-3-positive oligodendrocytes in the WM in each section were counted and averaged.

Western blot

Whole lysates from spinal cord (1 cm) were prepared with a lysis buffer containing 1% Nonidet P-40, 20 mM Tris, pH 8.0, 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 10 mM $Na_2P_2O_7$, 10 mM NaF, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium vanadate, and 1 mM PMSF. The protein concentration was determined using BCA assay kit (Pierce, Rockford, IL). Protein samples (40 μg each) were separated on SDS-PAGE and transferred to nitrocellulose membrane (Millipore). The membranes were blocked in 5% non-fat skim milk or 5% bovine serum albumin in TBS-T (0.1% Tween 20) for 1 h at room temperature followed by incubation with antibodies against β-Tubulin (1:10,000, Sigma St. Louis, MO), p38MAPK (1:1000, Cell Signaling Technology), p-p38MAPK (1:1,000, Cell Signaling Technology), cleaved caspase-3 (1:1000, Cell Signaling Technology), iNOS (1:1000, Transduction Laboratory, Lexington, KY), COX-2 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), and proNGF (1:1000, Alomone Labs, Jerusalem, Israel) at 4 °C overnight. After washing, the membranes were incubated with HRP conjugated secondary antibodies (Jackson ImmunoResearch) for 1 h. Immunoreactive bands were visualized by chemiluminesence using Supersignal (Pierce). β-Tubulin was used as an internal control. Relative intensity (relative to sham value) of each band on Western blots was measured and analyzed by AlphaImager software (Alpha Innotech Corporation, San Leandro, CA). Background in films was subtracted from the optical density measurements. Experiments were repeated three times, and the values obtained for the relative intensity were subjected to statistical analysis. The blots shown in figures are representative of results from three separate experiments.

RNA isolation and RT-PCR

RNA isolation using TRIZOL Reagent (Invitrogen) and cDNA synthesis were performed as previously described (Lee et al., 2003). A 20 μl PCR reaction contained 1 μl first strand cDNA, 0.5 U taq polymerase (Super Bio, Suwon, Korea), 20 mM Tris–HCl, pH 7.9 100 mM KCl, 1.5 mM $MgCl_2$, 250 μM dNTP, and 10 pmol, of each specific primer. Samples were subjected to 25–30 cycles of 95 °C 30 s, 50–60 °C 30 s, and 72 °C 30 s on a thermocycler (PerkinElmer, Emeryville, CA). The primers used for tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, COX-2, iNOS (Lee et al., 2003), matrix metalloprotease (MMP)-2, MMP-9 and GAPDH (Park et al., 2007) were synthesized by the Genotech (Daejeon, Korea). The sequences of the primers were: 5'-CCC AGA CCC TCA CAC TCA GAT-3' (sense) and 5'-TTG TCC CTT GAA GAG AAC CTG-3' (antisense) for TNF-α; 5'-GCA GCT ACC TAT GTC TTG CCC GTG-3' (sense) and 5'-GTC GTT GCT TGT CTC TCC TTG TA-3' for IL-1β; 5'-AAG TTT CTC TCC GCA AGA TAC TTC CAG CCA-3' (sense) and 5'-AGG CAA ATT TCC TGG TTA TAT CCA GTT T-3' (antisense) for IL-6; 5'-CTC CAT GAC TCT CAG CAC AGA G-3' (sense) and 5'-GCA CCG AAG ATA TCC TCA TGA T-3' (antisense) for iNOS; 5' CCA TGT CAA AAC CGT GGT GAA TG-3' (sense) and 5'-ATG GGA GTT GGG CAG TCA TCA G-3' (antisense) for COX-2; 5'-ACC ATC GCC CAT CAT CAA GT-3' (sense) and 5'-CGA GCA AAA GCA TCA TCC AC-3' (antisense) for MMP-2; 5'-AAA GGT CGC TCG GAT GGT TA-3' (sense) and 5'-AGG ATT GTC TAC TGG AGT CGA-3' (antisense) for MMP-9; 5'-TCC CTC AAG ATT GTC AGC AA-3' (sense) and 5'-AGA TCC ACA ACG GAT ACA TT-3' (antisense) for GAPDH, which was used as an internal control. After amplification, PCR products were subjected to a 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The relative density of bands (relative to sham value) was analyzed by the AlphaImager software (Alpha Innotech). Experiments were repeated three times and the values obtained for the relative intensity were subjected to statistical analysis. The gels shown in figures are representative of results from three separate experiments.

Quantitation of the proportion of resting and activated microglia

Percentage of field analysis was used to provide a quantitative estimate (proportional) of changes in the activation state of microglia. Resting and activated microglia were classified and counted based on a previous report (Hains and Waxman, 2006). Briefly, using immunostaing with OX-42 antibody (Millipore), resting microglia displayed small compact somata bearing long, thin, and ramified processes. Activated microglia exhibited marked cellular hypertrophy and retraction of processes such that the process length was less than the diameter of the soma compartment. Cells were sampled only if the nucleus was visible within the plane of section and if cell profiles exhibited distinctly delineated borders (Hains and Waxman, 2006).

Gelatin zymography

The gelatinase levels of MMP-2 and MMP-9 in the injured spinal cord were examined 1 day postinjury by gelatin zymography based on a previously described protocol with some modifications (Hsu et al., 2008; Svedin et al., 2007). Whole tissue lysates were prepared as described above. After determination of protein concentration of the homogenates, equal amounts of protein (50 μg) were loaded on a Novex 10% zymogram gel (EC61752; Invitrogen) and separated by electrophoresis with 100 V (19 mA) at 4 °C for 6 h. The gel was then incubated with renaturing buffer (2.5% Triton X-100) at room temperature for 30 min to restore the gelatinolytic activity of the proteins. After incubation with developing buffer (50 mM Tris–HCl, pH 8.5, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brii35) at 37 °C for 24 h, the gel

was stained with 0.5% Coomassie blue for 60 min and then destained with 40% methanol containing 10% acetic acid until appropriate color contrast was achieved. Clear bands on the zymogram were indicative of gelatinase activity. Relative intensity of zymography (relative to sham value) was measured and analyzed by AlphaImager software (Alpha Innotech). Background in films was subtracted from the optical density measurements. Experiments were repeated three times and the values obtained for the relative intensity were subjected to statistical analysis.

Behavioral tests

Examination of functional deficits after injury was conducted as previously described (Yune et al., 2007). Behavioral analyses were performed by trained investigators who were blind as to the experimental conditions. To test hindlimb locomotor function, open-field locomotion was evaluated by using the Basso–Beattie–Bresnahan (BBB) locomotion scale as previously described (Basso et al., 1995; Yune et al., 2007). BBB is a 22-point scale (scores 0–21) that systematically and logically follows recovery of hindlimb function from a score of 0, indicative of no observed hindlimb movements, to a score of 21, representative of a normal ambulating rodent. Inclined plane test was performed by the method described previously (Rivlin and Tator, 1977). In brief, animals were tested in two positions (right side or left side up) on the testing apparatus (i.e., a board covered with a rubber mat containing horizontal ridges spaced 3 mm apart). The maximum angle at which a rat could maintain its position for 5 s without falling was recorded for each position and averaged to obtain a single score for each animal. The ability to control and place the hindlimb precisely was tested on a horizontal grid as previously described (Merkler et al., 2001). Analysis was performed by counting the number of foot fall (mistake) in foot placing. Footprint analysis was performed as previously described (Stirling et al., 2004). Both animal's forepaws and hindpaw were dipped in red and blue dye (non toxic) and then walked across a narrow box (1 m long and 7 cm wide). The footprints were scanned, and digitized images were analyzed.

Axon staining and counting

The rats of control and acupuncture-treated groups were anesthetized at 38 days after injury, and frozen sections were prepared as described above. For quantitative analysis of axonal densities, serial coronal sections collected every millimeter rostral and caudal 3 mm to the lesion site were stained with an antibody specific for 200 kDa neurofilament protein (NF200; 1:4000, Sigma). Some sections were processed for 5-hydroxytryptamine (5-HT; 1:5000, Diasorin, Stillwater, MN) staining. The ABC method was used to detect labeled cells using a Vectastain kit (Vector Laboratories, Burlingame, CA). Axonal densities were determined within preselected fields (40×40 μm, 1600 μm^2) at specific sites within the vestibulospinal tract (VST) for NF200-positive axons as previously described (Yune et al., 2007). The location of these sites was carefully conserved from group to group using anatomical landmarks, and remaining axons were manually counted from each field. The number of axons in control or acupuncture-treated spinal cord was expressed as a percentage relative to that in sham control (100%).

Assessment of lesion volume

The measurement of lesion volume using rats tested for behavioral analysis was performed as previously described (Yune et al., 2008). Serial longitudinal sections (10 μm) through the dorsoventral axis of the spinal cord were used to determine lesion volume. Every 50 μm section was stained with Cresyl violet acetate and was studied with light microscopy. The rostrocaudal boundaries of the tissue damage were defined by the presence of inflammatory cells, the loss of neurons, the existence of degenerating neurons and cyst formation. With a low-power ($1.25\times$) objective, the lesion area was determined by MetaMorph software (Molecular devices). Areas at each longitudinal level are determined, and the total lesion volume is derived by means of numerical integration of sequential areas.

Statistical analysis

Data are presented as mean ± SD values. Comparisons between vehicle and acupuncture-treated groups were made by unpaired Student's t test. Muptiple comparisons between groups were performed one-way ANOVA. Behavioral scores from BBB analysis and inclined plane tests were analyzed by repeated measures ANOVA (time vs treatment). Tukey's multiple comparison was used as Post hoc analysis. Statistical significance was accepted with $p<0.05$. All statistical analyses were performed by using SPSS 15.0 (SPSS Science, Chicago, IL).

Results

Acupuncture inhibits apoptotic cell death of neurons and oligodendrocytes after SCI

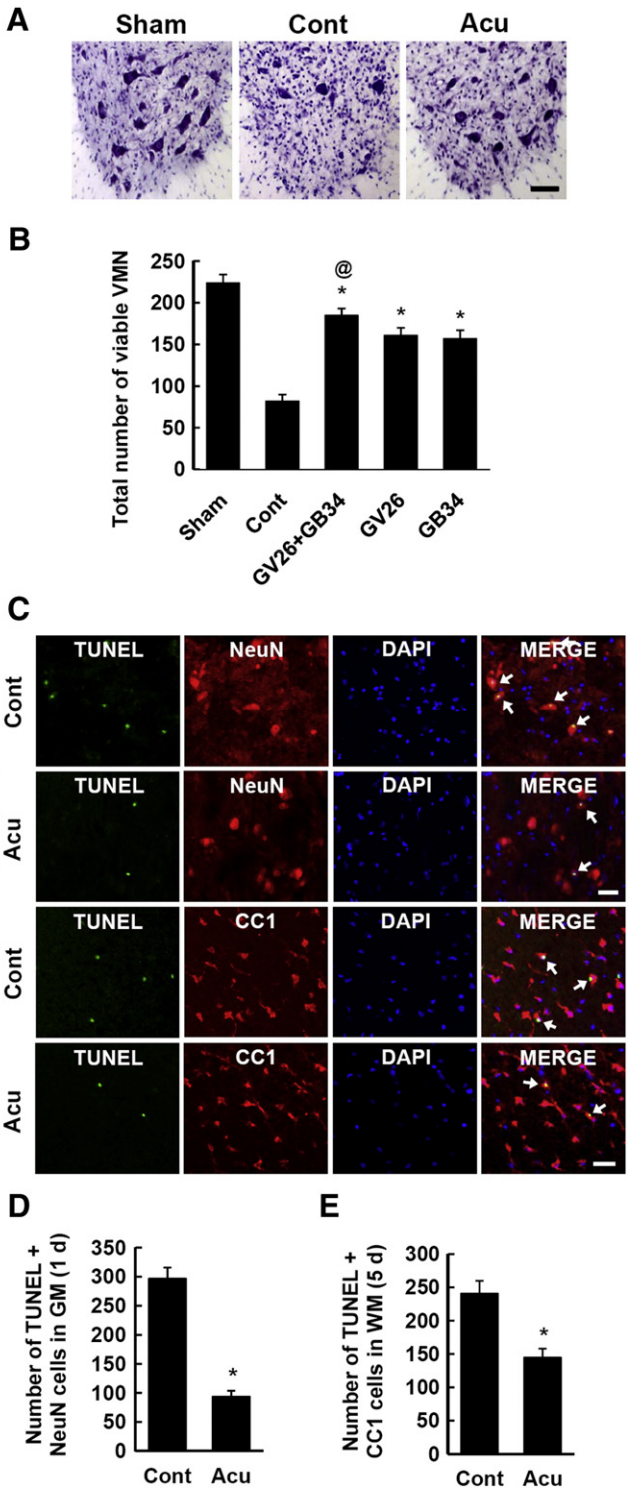
Trauma to the spinal cord results in extensive apoptotic cell death (Lee et al., 2003; Liu et al., 1997). First, we examined the effect of acupuncture treatment on the number of viable motor neurons in the ventral horn at 4 days after injury. As shown in Fig. 2A (Cont), a massive loss of ventral motor neurons (VMN) was observed in the lesion area after injury, as reported (Yune et al., 2008). However, acupuncture treatment significantly alleviated VMN loss in both the rostral and caudal to the lesion epicenter when compared with control (Sham, 224 ± 10; Cont, 82 ± 7.7; GV26 plus GB34, 185 ± 8.2; GV26, 161 ± 9; GB34, 157 ± 10 cells; Cont vs GV26 plus GB34, GV26, or GB34, $p<0.001$) after injury (Fig. 2A, B). Also, VMN loss was more mitigated by acupuncture at both GV26 and GB34 acupoints together than at each acupoint, GV26 or GB34 alone applied ($p<0.05$) (Fig. 2B). Thus, we used the two specific acupoints, GV26 and GB34, throughout our experiments. To confirm the specific effect of acupuncture, we treated injured rat with a simulated acupuncture with a toothpick as described by Cherkin et al. (2009) and examined the VMN loss at 4 days after injury. As shown in Fig. 3A, VMN loss was not alleviated by a simulated acupuncture treatment whereas real acupuncture treatment significantly reduced VMN loss after injury (Sham, 235 ± 9; Cont, 85 ± 9.3; GV26 plus GB34, 189 ± 8.2; Sim-GV26, 81 ± 9; Sim-GB34, 87 ± 11; Sim-GV26 plus GB34, 77 ± 13 cells; Cont vs GV26 plus GB34, $p<0.01$). Thus, these data indicate that a simulated acupuncture treatment has no neuroprotective effect on VMN loss after SCI. We also found that there was no significant protective effect on VMN loss after injury when acupuncture was applied at a site 2 mm from each specific acupoint, GV26 or GB34 (data not shown). In addition, we observed short and mild, hindlimb flexion when acupuncture was applied at the hindlimb within 3–4 days after injury. However, this phenomenon was not observed in simulated acupuncture-treated and sham control rats.

Next, we examined whether acupuncture treatment (GV26 and GB34) would reduce apoptotic cell death after SCI by counting TUNEL-positive neurons (NeuN) in the GM (1 day) and oligodendrocytes (CC1) in the WM (5 days) after injury. As shown in Fig. 2C, a number of TUNEL-positive neurons and oligodendrocytes were observed in the GM (1 day) and WM (5 days), respectively, consistent with previous reports (Stirling et al., 2004; Yune et al., 2003, 2007). Quantitative analysis also revealed that acupuncture treatment led to a significant reduction in the number of TUNEL-positive neurons and oligodendrocytes when compared to the control group (neurons, Acu 94 ± 10 vs Cont 297 ± 23 cells; oligodendrocytes, Acu 145 ± 13 vs

Cont 241 ± 19 cells, $p<0.001$) (Fig. 2D). Our results thus indicate that acupuncture alleviates apoptotic cell death following SCI.

Acupuncture inhibits caspase-3 activation after SCI

Caspase-3 activation occurs at an early stage of apoptotic cell death after SCI (Citron et al., 2000; Springer et al., 1999). Since we demonstrated that acupuncture inhibited neuronal and oligodendroglial apoptosis after SCI (see Fig. 2), we anticipated that acupuncture treatment would inhibit caspase-3 activation after injury. Caspase-3 was activated at early (4 h) and delayed time (5 days) points after injury as reported (Springer et al., 1999; Yune et al., 2007). Acupuncture treatment significantly decreased the levels of cleaved (activated) forms of caspase-3 at 4 h and 5 days after injury when compared those in control (Fig. 4A, B). Double labeling also revealed that a number of activated caspase-3-positive oligodendrocytes at 5 days after



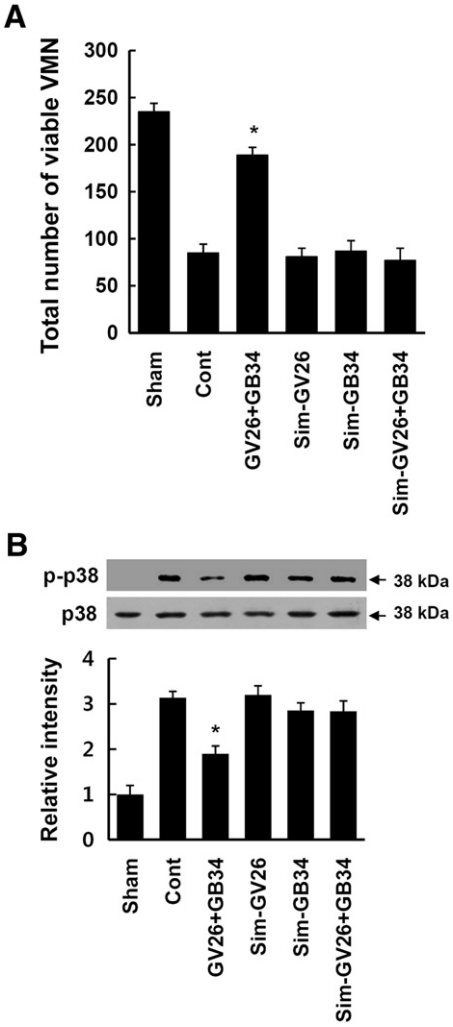


Fig. 3. The effect of simulated acupuncture treatment on VMN loss and p38MAPK activation after SCI. Injured rats treated with simulated acupuncture as described in the Methods section and total spinal extracts were prepared at 4 and 5 days after injury $n=3$/group). A, Quantification of viable VMN at 4 days and B, Western blot of p-p38MAPK at 5 days after injury. Data are presented as means ± SD from three separate experiments.

Fig. 2. Acupuncture inhibits apoptotic cell death after SCI. A. Ventral horn areas displaying viable motor neurons (VMN) in the sham, control and acupuncture-treated groups after injury. Representative sections were taken 2 mm rostral to the lesion epicenter at 4 days after injury. Scale bar, 40 μm. B. Quantitative analysis of viable motor neurons in sham, control and acupuncture-treated groups after injury ($n=5$/group). Data are presented as mean ± SD from five separate experiments. $^{*}p<0.001$ compared to control; $^{@}p<0.05$ compared to GV26 or GB34. For TUNEL staining, spinal cord samples at 1 or 5 days after injury were collected and processed for TUNEL staining, and TUNEL-positive neurons and oligodendrocytes were counted as described in the Methods section. C. (upper panels) TUNEL-positive neurons (NeuN) (arrows) in the ventral horn area at 1 day after injury. Representative images were from the sections selected 1 mm rostral to the lesion epicenter. C. (bottom panels) TUNEL-positive oligodendrocytes (CC1) (arrows) in the WM at 5 days after SCI. Representative images were from the sections selected 5 mm rostral to the lesion epicenter. Scale bars, 30 μm. Quantitative analysis of TUNEL-positive neurons at 1 day (D) or oligodendrocytes at 5 days (E) ($n=5$/group). Data are presented as means ± SD from five separate experiments. $^{*}p<0.001$.

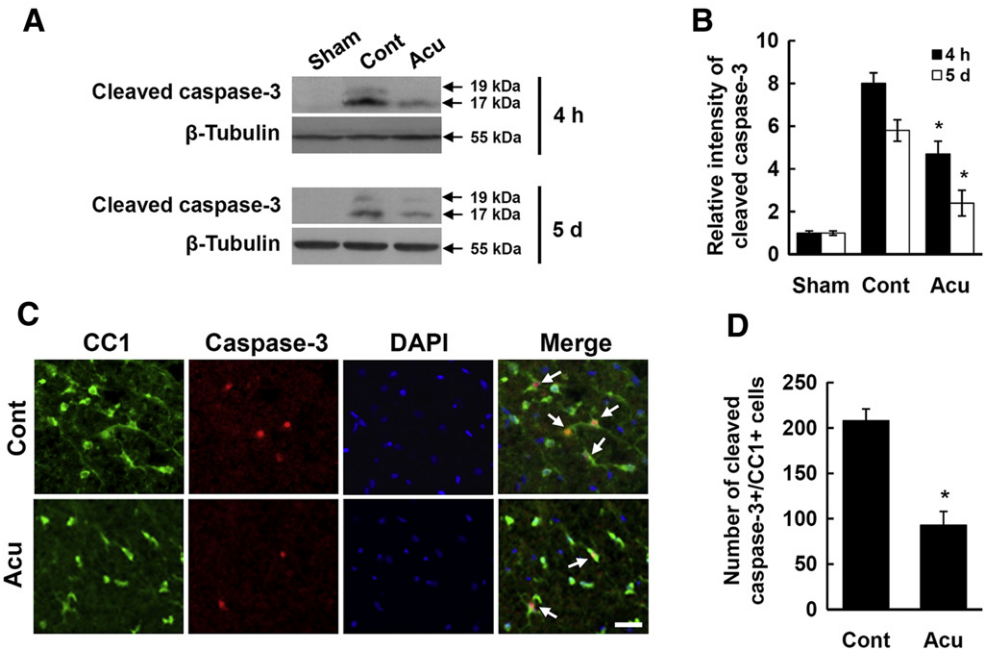


Fig. 4. Acupuncture inhibits activation of caspase-3 after SCI. (A) Western blots analysis of activated caspase-3 at 4 h and 5 days after injury. (B) Quantitative analysis of Western blots ($n=3$/group). All data are presented as means ± SD of three separate experiments. *$p<0.05$. (C) Activated capase-3 positive oligodendrocytes (arrows) in control and acupuncture-treated spinal cord at 5 days after injury. Representative images were from the sections selected 5 mm rostral to the lesion epicenter. Scale bar, 30 μm. (D) Quantitative analysis of activated caspase-3-positive oligodendrocytes (CC1) at 5 days after injury ($n=5$/group). Cleaved caspase-3-positive oligodendrocytes were counted as described in the Methods section. Data are presented as means ± SD from five separate experiments. *$p<0.05$.

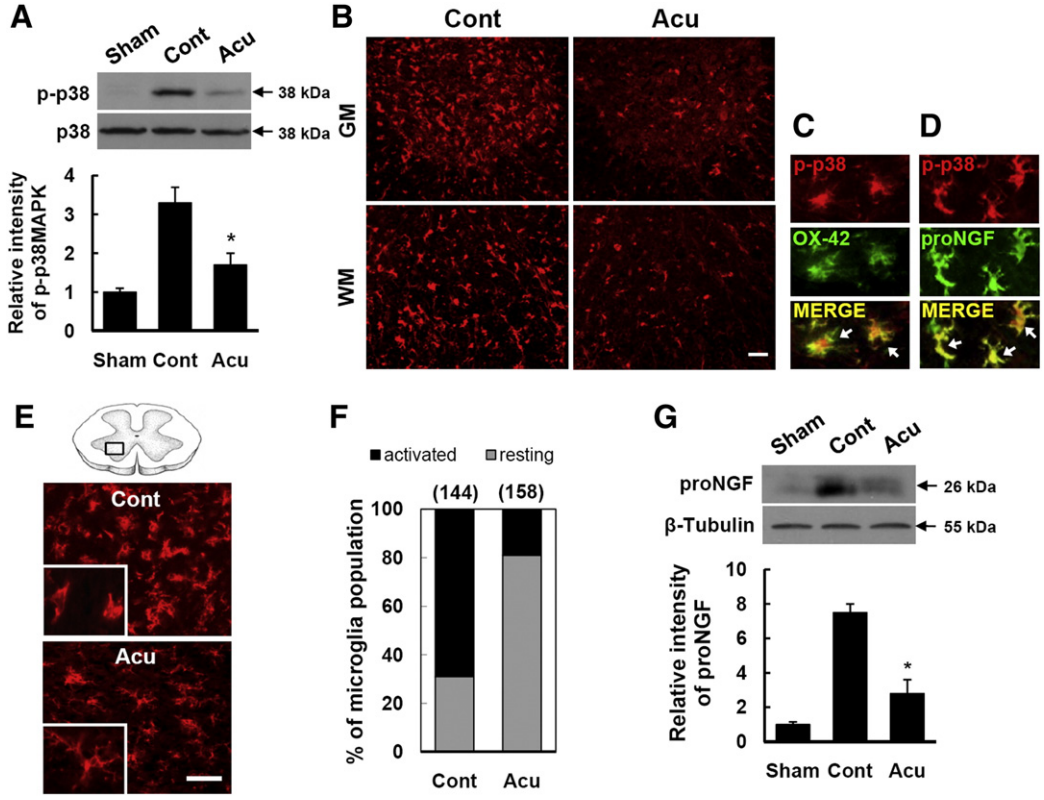


Fig. 5. Acupuncture inhibits p38MAPK activation in microglia after SCI. A. Western blot of p-p38MAPK at 5 days after injury (upper panel) and quantitative analysis of Western blots (lower panel, $n=3$/group). Values are presented as means ± SD of three separate experiments. *$p<0.001$. B. Immunostaining showing the presence of p-p38MAPK-positive microglia in the GM and the WM at 5 days after injury. Representative images are from sections 4 mm caudal to the lesion epicenter. Scale bar, 50 μm. C. OX-42-positive microglia were positive for p-p38MAPK (arrows) after SCI. D. ProNGF was expressed in p-p38MAPK-positive microglia (arrows). E. OX-42-positive microglia in the ventral horn (upper panel, box) at 5 days after injury. Sections were taken 2 mm caudal to the lesion epicenter. Activated microglia exhibit marked cellular hypertrophy and retraction of processes after injury (Cont, insert), while resting microglia display smaller somata with processes (Acu, insert). Scale bar, 50 μm. F. Quantification of the proportion of resting and activated microglia ($n=5$/group). The number of resting or activated microglia in control or acupuncture-treated spinal cord was expressed as a percentage of total cells (100%) sampled. Data are presented as means from five separate experiments. Parentheses indicate the number of microglia sampled. G, Western blot of proNGF at 5 days after injury (upper panel) and quantitative analysis of Western blots (lower panel, $n=3$/group). Values are presented as means ± SD of three separate experiments. *$p<0.001$.

injury were observed in the WM as reported (Fig. 4C) (Yune et al., 2007). However, acupuncture treatment significantly reduced the number of activated caspase-3-positive oligodendrocytes as compared with control (Cont 208 ± 13 vs Acu 93 ± 15, $p<0.05$) (Fig. 4D).

Acupuncture inhibits activation of p38MAPK and proNGF expression in microglia after SCI

It is known that p38MAPK mediates inflammatory responses in microglia (Bhat et al., 1998). Also, our previous report shows that oligodendroglial apoptosis is mediated by proNGF production via activation of p38MAPK in microglia after SCI (Yune et al., 2007). Since inflammation induces neuronal and oligodendroglial apoptosis (Bao and Liu, 2004; Bareyre and Schwab, 2003) and acupuncture reduced apoptotic cell death after injury (see Fig.. 2), we hypothesized that acupuncture would inhibit p38MAPK activation and proNGF expression in microglia after SCI. As shown in Fig. 5A, acupuncture treatment significantly decreased the level of p38MAPK activation after injury as compared to control. Again, to confirm the specific effect of acupuncture, we treated injured rats with simulated acupuncture with a toothpick as described by Cherkin et al. (2009). As shown in Fig. 3B, the level of p-p38MAPK was not significantly decreased by a simulated acupuncture treatment as compared to control. Next, the numbers of p-p38MAPK-positive cells in the GM and the WM also were markedly lower in the acupuncture-treated group than the control group (Fig. 5B). In addition, OX-42-positive microglia were positive for p-p38MAPK (Fig. 5C) and p-p38MAPK-positive microglia also expressed proNGF after SCI (Fig. 5D) as reported (Yune et al., 2007). Immunohistochemistry using an antibody against OX-42 revealed that microglia exhibited an activated phenotype after SCI, including marked cellular hypertrophy and retraction of cytoplasmic processes (Fig. 5E, insert), as reported (Hains and Waxman, 2006). Acupuncture treatment significantly decreased the proportion of activated microglia (determined by counting the number of cells with processes longer/shorter than the soma diameter) as compared with control ($p<0.01$) (Fig. 5F). Furthermore, the level of proNGF was significantly lower in the acupuncture-treated group than that in the control group (Fig. 5G). Thus, these data indicate that acupuncture inhibits microglial activation after SCI.

Acupuncture inhibits expression of proinflammatory cytokines and mediators after SCI

Our previous reports show that after SCI, activated microglia produce proinflammatory cytokines and inflammatory mediators, which induce cell death of neurons and oligodendrocytes (Lee et al., 2003; Yune et al., 2007). Since we showed that acupuncture inhibited neuronal and oligodendroglial apoptosis after injury (see Fig. 2), we hypothesized that acupuncture would inhibit expression of proinflammatory cytokines and mediators after SCI. RT-PCR and quantitative analysis data revealed that acupuncture treatment significantly inhibited expression of TNF-α, IL-1β, IL-6, COX-2 and iNOS when compared to those in the control group (Fig. 6A, B). Western blot analysis also revealed that acupuncture significantly decreased the levels of the inflammatory mediators, COX-2 and iNOS, as compared to those in control (Fig. 6C, D).

Acupuncture inhibits MMP-9 expression and activation after SCI

It has been shown that MMP-9 plays an important role in inflammation and blood–spinal cord barrier dysfunction, and its expression is rapidly increased after SCI (Noble et al., 2002). Since acupuncture inhibited microglial activation (see Figs. 5 and 6), we hypothesized that acupuncture would reduce MMP-9 expression after SCI. To examine the effect of acupuncture treatment on MMPs expression and activation at 1 day after injury, we performed RT-PCR and zymography. As shown in Fig. 7, the levels of mRNA expression and enzymatic activation of MMP-2 and MMP-9 were significantly increased after injury as reported (Hsu et al., 2008; Noble et al., 2002; Wells et al., 2003). Furthermore, acupuncture treatment significantly decreased the levels of mRNA expression and enzymatic activation of MMP-9 (e.g., enzymatic activity; Acu 2.2 ± 0.25 vs Cont 4.9 ± 0.32, $p<0.01$) when compared to control (Fig. 7A–D). By contrast, the levels

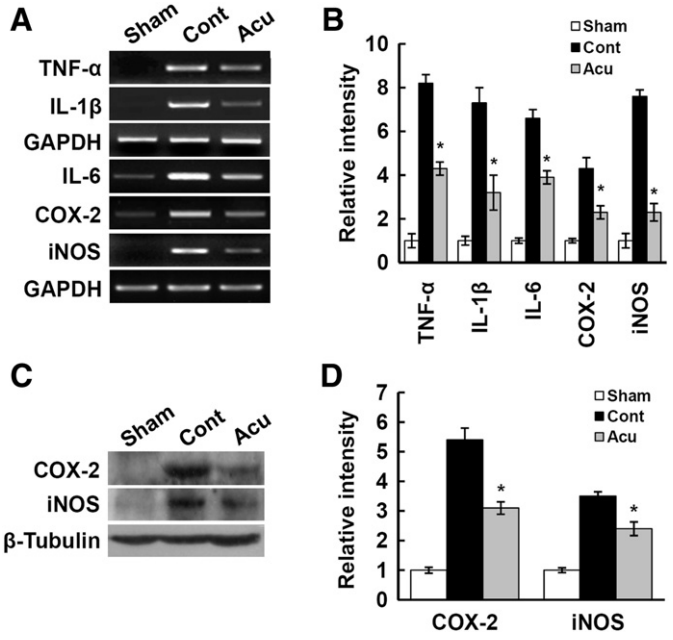


Fig. 6. Acupuncture inhibits the expression of proinflammatory factors after SCI. Total RNA and protein extracts from spinal cord samples at 1 h (for TNF-α and IL-1β) and 6 h (IL-6, COX-2 and iNOS) after injury were prepared, as described in the Methods section. A. RT-PCR of TNF-α, IL-1β, IL-6, COX-2 and iNOS. B. Quantitative analysis of RT-PCR ($n=3$/group). *$p<0.01$. C. Western blot of COX-2 and iNOS. D. quantitative analysis of Western blots ($n=3$/group). Data are presented as means ± SD from three separate experiments. *$p<0.001$.

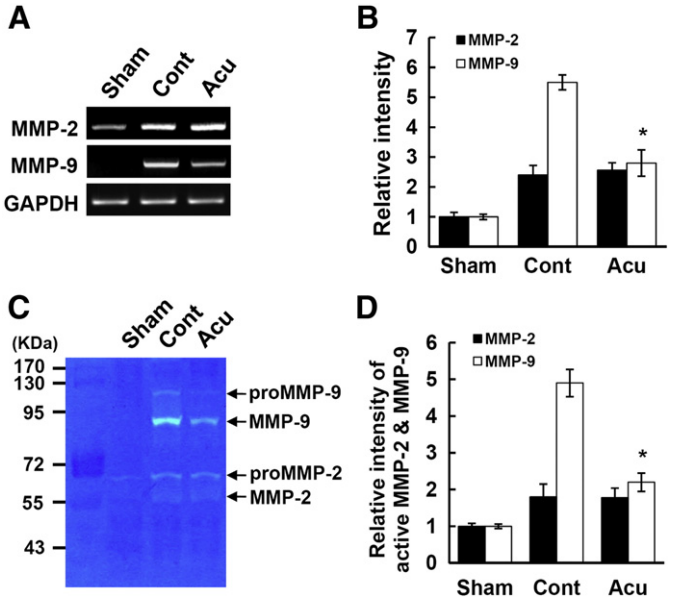


Fig. 7. Acupuncture treatment inhibits MMP-9 expression and activation after SCI. Total RNA and protein extracts from spinal cord samples at 1 day after injury were prepared, as described in the Methods section. RT-PCR of MMP-2 and -9 (A) and quantitative analysis (intensity relative to sham value) of RT-PCR (B) ($n=3$/group). *$p<0.05$. Zymography of MMP-2 and -9 (C) and quantitative analysis (intensity relative to sham value) of zymography (D) ($n=3$/group). Data are presented as means ± SD from three separate experiments. *$p<0.01$.

of mRNA expression and enzymatic activation of MMP-2 were not affected by acupuncture treatment (Fig. 7A–D).

Acupuncture improves functional recovery after SCI

Since acupuncture treatment inhibited apoptosis after SCI (see Fig. 2), we hypothesized that acupuncture would improve functional recovery after injury. Rats receiving the 25 mm insult were subjected to acupuncture at two acupoints, GV26 and GB34, as described in the Methods section ($n=25$/group). Functional recovery was evaluated for 35 days after injury using the BBB rating scale (Basso et al., 1995), grid walk test (Merkler et al., 2001), incline test (Rivlin and Tator, 1977) and footprint analysis (Stirling et al., 2004). BBB scores were significantly higher in rats treated with acupuncture at 21–35 days after SCI than control rats (35 days, Acu 11.7 ± 0.6 vs Cont 9.3 ± 0.4, $p<0.01$) (Fig. 8A). The ability to control and place the hindlimbs precisely was examined on a horizontal grid at 35 days after injury. As shown in Fig. 8B, the number of mistakes (footfalls on the grid walk) in the acupuncture-treated group was significantly lower than that observed in the control group (Acu 2.33 ± 0.43 vs Cont 9.33 ± 0.77 mistakes, $p<0.05$). The angle of incline, determined 1–4 weeks after injury, was also significantly higher in acupuncture-treated rats (4 weeks, Acu 64.3 ± 2.0 vs Cont 56.9 ± 2.1, $p<0.05$) (Fig. 8C). Finally, footprint analyses obtained from control and acupuncture-treated rats at 35 days after SCI disclosed fairly consistent forelimb-hindlimb coordination and very little toe dragging in the acupuncture-treated group, in contrast to consistent dorsal stepping and extensive drags in control rats, as revealed by ink streaks extending from both hindlimbs (Fig. 8D). With the 25 mm insult applied in the present study, hindpaws and central pads of control animals were not clearly recorded due to hindlimb drags (Fig. 8D). Thus, we were unable to quantify footprint analysis using toe spread and ipsilateral distances (limb coordination).

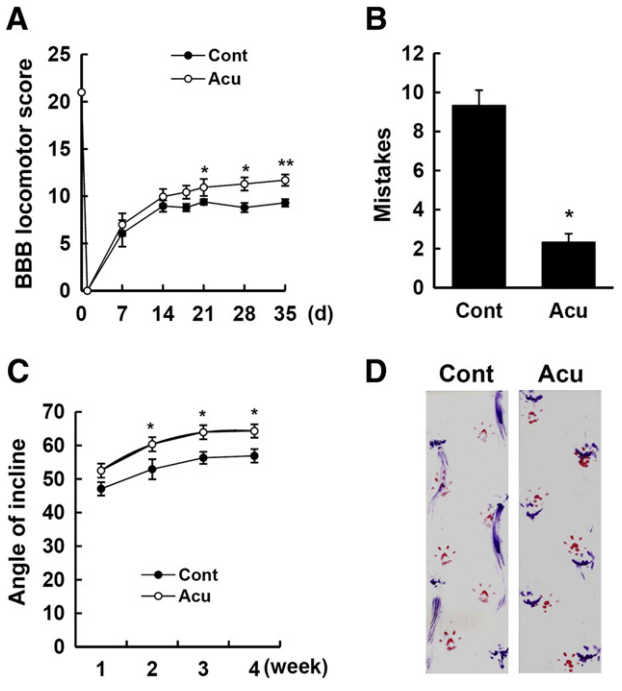


Fig. 8. Acupuncture treatment improves functional recovery after SCI. Functional recovery was assessed with the BBB test, grid walk test, inclined plane test, and footprint analysis after injury. Each value represents the mean ± SD obtained from 25 animals. A. BBB scores of vehicle- and acupuncture-treated groups after injury. $^{*}p<0.05$, $^{**}p<0.001$. B. Inclined plane test of vehicle- and acupuncture-treated groups after injury. $^{*}p<0.05$. C. Grid walk test of vehicle- and acupuncture-treated groups at 38 days after injury. $^{*}p<0.05$. D. Representative footprints obtained from each group at 35 days after SCI.

Acupuncture reduces axon loss and lesion volume after SCI

Functional deficits after SCI are correlated with greater axon loss in the WM (Basso et al., 1996). To examine whether acupuncture preserves axons after injury, immunostaining with NF200 and 5-HT antibodies was performed to detect the remaining axons, and the density of preserved axons was counted, as described in the Methods section. In sham control animals, NF200-positive axons within the VST were dense, and axonal packing was uniform (Fig. 9A, Sham). After SCI, axon density was markedly decreased and exhibited patchy distribution in injured tissue at 38 days after injury (Fig. 9A Cont). Quantitative analysis revealed that the number of NF200-positive axons in the VST was significantly higher in the acupuncture-treated group as compared to the control group (2 mm, Acu 41 ± 5 vs Cont 26 ± 3%; 3 mm, Acu 62 ± 4.6 vs Cont 33 ± 4%, $p<0.05$) (Fig. 9B). Moreover, the density of 5-HT serotonergic axons in the ventral horn was higher in the acupuncture-treated group than the control group (Fig. 9C). These results indicate that acupuncture treatment alleviates axon loss after SCI.

Traumatic injury to the spinal cord triggers immediate mechanical damage, followed by a secondary cascade of degenerative processes, leading to progressive tissue loss (Lee et al., 2003; Schwab and Bartholdi, 1996). To evaluate whether acupuncture reduces tissue loss after SCI, serial longitudinal sections from acupuncture-treated and control spinal cords were cut and stained with Cresyl violet acetate. Extension of the cystic cavity in the lesion site was observed at 38 days after injury (Fig. 9D). The total lesion volume was significantly decreased upon acupuncture treatment as compared to that of control (Acu 4.1 ± 1.0 vs Cont 9.9 ± 1.2 mm3, $p<0.05$), at 38 days after injury (Fig. 9E).

Discussion

We demonstrated for the first time the therapeutic efficacy of acupuncture leading to a partial functional recovery in an animal model of SCI. Our data showed that acupuncture treatment provided a significant neuroprotection against moderate spinal contusion injury in the rat. Acupuncture reduced apoptotic cell death of both neurons and oligodendrocytes and thereby improved functional recovery after SCI. Also, acupuncture decreased p38MAPK activation and expression of proinflammatory cytokines/mediators and proNGF, which are involved in apoptotic cell death of neurons and oligodendrocytes after SCI as reported (Lee et al., 2003; Yune et al., 2007). In particular, acupuncture reduced the number of activated microglia after injury. Thus, our results show that the neuroprotective effects of acupuncture are mediated in part by alleviating neuronal and oligodendroglial apoptosis via inhibition of microglial activation after SCI.

We first tested and selected acupoints exerting the most effective neuroprotective effects by counting the number of viable VMN after SCI (see Fig. 1). Two acupoints, GV26 and GB34, were identified as the most neuroprotective acupoints after injury. Furthermore, we found that acupuncture applied simultaneously at GV26 and GB34 acupoints was more effective than a separate stimulation at each acupoint. GB34 is located at the point of intersection of lines from the anterior border to the head of the fibula (Yin et al., 2008) and has been used traditionally and clinically to treat patients with PD-like symptoms (Park et al., 2003). GV26, located at the midpoints between base of the columnar nasi and the upper lip, on the facial midline (Yin et al., 2008), has been applied for the treatment in transient middle cerebral artery occlusion (MCAO) rats (Gan et al., 2005; Wang et al., 2002; Zhao et al., 2007). In particular, electroacupuncture (EA) at GV26 inhibits apoptotic cell death of neurons in the cerebral cortex after MCAO by potentiating Akt , serine/threonine protein kinase B, and suppressing caspase-9 activation (Wang et al., 2002). EA at GV26 in MCAO rats also decreases the ischemic damaged area in the cerebral cortex and hippocampus (Gan et al., 2005). To confirm the specific

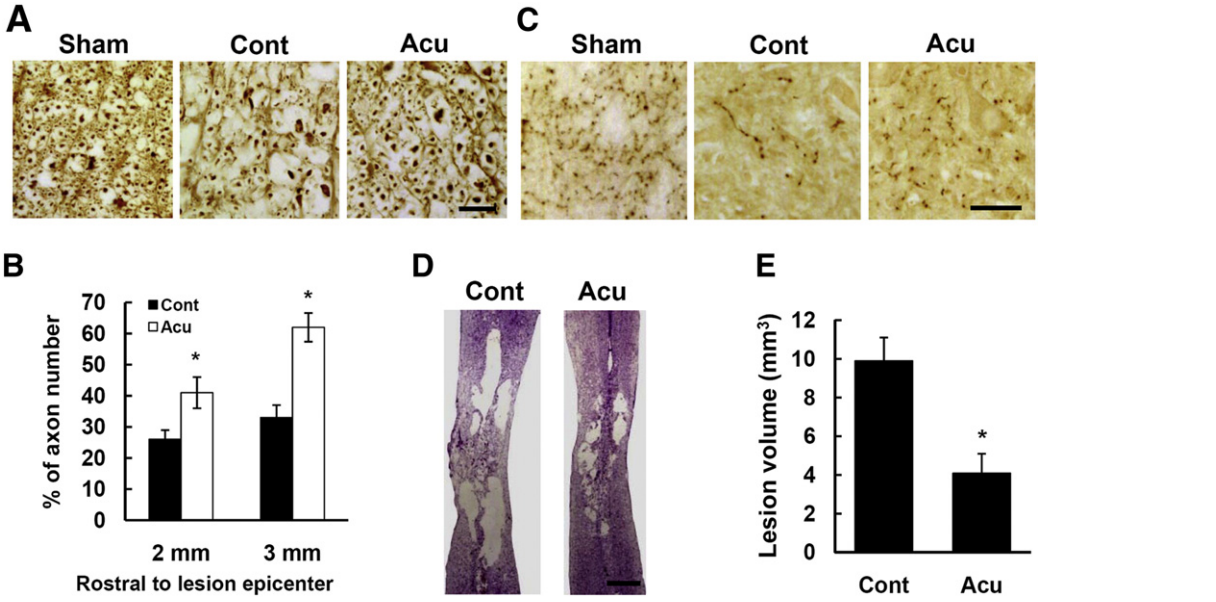


Fig. 9. Acupuncture treatment inhibits axon loss and reduces lesion volume after SCI. Transverse sections taken from 3 mm rostral and caudal to the lesion epicenter at 38 days after injury were processed for NF200 or 5-HT staining. A. Representative photographs of NF200-positive axons in sham, control and acupuncture-treated spinal sections 3 mm rostral to the lesion epicenter. B. Quantification of the remaining NF200-positive axons within preselected fields (40×40 μm, 1600 μm^2) at specific sites within vestibulospinal tract ($n=5$/group). Scale bar, 20 μm. Data are presented as means ± SD from five separate experiments. *$p<0.05$. C. Representative photographs of 5-H2T-postive axons in ventral horn areas in sections 3 mm caudal to the lesion site. Scale bar, 30 μm. D. Representative spinal cord tissues (1.2 mm from dorsal surface) showing cavitation in the lesion site at 38 days after injury. Scale bar, 1 mm. E. Quantitative analysis of lesion volumes at 38 days after injury ($n=5$/group). Data are presented as means ± SD from five separate experiments. *$p<0.05$.

effect of acupuncture, we examined the effect of simulated acupuncture using a toothpick (Cherkin et al., 2009) and found that both VMN loss and p38MAPK activation were not inhibited by a simulated acupuncture treatment (see Fig. 3). Taken together, these results suggest that acupuncture at both GB34 and GV26 may provide neuroprotection in neurodegenerative diseases and in CNS injuries.

SCI-induced and delayed apoptotic cell death of neurons and oligodendrocytes has been known to cause progressive degeneration of the spinal cord, leading to permanent functional deficits (Beattie et al., 2002; Crowe et al., 1997; Liu et al., 1997; Yune et al., 2007). However, no therapeutic agents or tools are currently effective in inhibiting apoptotic cell death and restoring motor functions after SCI in human. Our data indicate that acupuncture treatment significantly inhibited apoptotic cell death of neurons and oligodendrocytes after SCI (see Fig. 2). Also, the size of lesion and the loss of axons after injury were significantly decreased following acupuncture treatment (see Fig. 9). To our knowledge, this is the first report showing that only acupuncture without the use of any drug is an effective therapeutic tool for recovery by inhibiting apoptosis after SCI.

Neuroprotection by acupuncture is documented in PD and ischemic animal models (Jang et al., 2003; Jeon et al., 2008; Park et al., 2003; Wang et al., 2003); however, the underlying mechanisms remain unclear. Our results indicate that acupuncture effectively attenuated microglial activation and reduced the expression of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, and inflammatory mediators, including iNOS, COX-2, and MMP-9 after injury (see Figs. 5–7). In particular, the activation of p38MAPK and the expression of proNGF, which occurred in activated microglia and involved in apoptotic cell death of oligodendrocytes after SCI (Yune et al., 2007) were significantly inhibited by acupuncture after injury (see Fig. 5). These findings are consistent with a recent report showing that acupuncture inhibits microglial activation and expression of inflammatory mediators in the MPTP mouse model of PD (Kang et al., 2007). Inflammation has been implicated in a number of neurodegenerative conditions, such as Alzheimer's disease (Hull et al., 2002), PD (McGeer et al., 2001) and brain ischemia (Liao et al., 2001). Following SCI, resident microglia are activated (Taoka and Okajima, 1998) and induce a rapid and robust upregulation of proinflammatory cytokines, including IL-1β, TNF-α, and IL-6 (Hayashi et al., 2000; Lee et al., 2003; Wang et al., 1996), thereby triggering apoptotic cell death. Our data show that the neuroprotective effects of acupuncture are likely mediated in part by inhibiting microglial activation as a result of injury.

Our results also indicate that acupuncture significantly attenuated MMP-9 expression and enzymatic activation after injury (see Fig. 7). MMPs play important roles in extracellular matrix remodeling, inflammation and wound healing (Yong et al., 2001). Among MMPs, MMP-9 is known to exert deleterious effects on recovery after SCI. For examples, MMP-9 is known to facilitate glial scar formation in the injured spinal cord (Hsu et al., 2008) and limit functional recovery by modulation of abnormal vascular permeability and inflammation (Noble et al., 2002). Therefore, our data suggest that neuroprotection by acupuncture may also be mediated in part by inhibiting MMP-9 activation after injury.

On the view of oriental medicine based on ancient theories, the effect of acupuncture is explained by the flow of the energy of the body, or Qi through these distinct channels/meridians that cover the body. According to this theory, acupuncture adjusts the flow of Qi in the body. In this way, acupuncture restores the harmonious balance of the body and its parts. However, these phenomena cannot be explained or proven scientifically at present. In our study, we found that the neuroprotective effect of acupuncture is likely mediated in part by inhibiting microglial activation and inflammatory responses after SCI, although the exact mechanisms underlying inhibition of microglial activation by acupuncture are not fully understood. It has been known that inflammation after SCI is related to blood infiltration such as neutrophil and macrophage (Donnelly and Popovich, 2008), increase of blood–spinal cord barrier permeability (Schnell et al., 1999), glutamate excitotoxicity (Byrnes et al., 2009), hypoxia-induced free radicals (Hausmann, 2003) and FAS ligand (CD95) (Demjen et al., 2004). Although we did not examine the effects of acupuncture on above mentioned mechanisms, we believe that these factors may be one of the targets for acupuncture-mediated action mechanisms. Therefore, further study for elucidating the mechanisms underlying acupuncture-mediated inhibition of inflammation after SCI is required. In addition, some papers showed that abolishing the immune response would be less effective than a fine tuning of the response in

treating SCI. For example, it has been reported that systemic inhibition of the immune responses by cyclosporine is less effective in functional recovery than vehicle-control (Ibarra et al., 2004). However, we are not able to speculate whether acupuncture affects systemic immune response and whether the effect of acupuncture on systemic immune response is beneficial or deleterious at present. Further study may be required to elucidate other possible mechanisms for acupuncture-mediated neuroprotection after SCI.

In summary, our data show that acupuncture treatment can improve functional recovery by reducing apoptotic cell death after SCI. In addition, the neuroprotection by acupuncture might be mediated in part by inhibiting microglial activation after injury. Furthermore, the present study suggests an application of acupuncture, especially at GV26 and GB34 acupoints, as a therapeutic tool in acute spinal injury patients.

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