Regulatory Effects of Samul-tang on Axonal Recovery after Spinal Cord Injury in Rats

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In oriental medicine, Samul-tang (SMT) has been used for the treatment of cardiovascular diseases and neuronal disorders. Here, possible effects of SMT on axonal regeneration after the spinal cord injury were examined. SMT treatment induced increases in regeneration-related proteins GAP-43, cell division cycle 2 (Cdc2) and phospho-Erk1/2 in the peripheral sciatic nerves after crush injury. Increased levels of Cdc2 and phospho-Erk1/2 were observe mostly in the gray matter area and some in the dorsomedial white matter. These increases correlated with increased cell numbers in affected areas. Moreover, axons of corticospinal tract (CST) showed increased sprouting in the injured spinal cord when administrated with SMT compared with saline-treated control. Thus, the present data indicate that SMT may be useful for identifying active components and for therapeutic application toward the treatment of spinal cord disorders after injury.

Key words : Samul-tang (SMT), regeneration, spinal cord injury, corticospinal tract, Cdc2, GAP-43, Erk1/2

Introduction

The spinal cord receives sensory information from the body and transmits to the brain, and at the same time, it controls body muscle by relaying motor command signals from the brain. Spinal cord lesion can cause either paraplegia (paralysis of the lower body) or quadriplegia (paralysis of the body from the neck) permanently. Unlike peripheral axons, axon tracts in the spinal cord after injury are not able to regenerate, and, in many cases, neuronal cell bodies corresponding to injured axons degenerate consequently resulting in the permanent loss of function[1-4]. Several studies suggest that central nervous system (CNS) neurons are intrinsically defective for new axon growth after injury because of the lack of intrinsic growth-promoting molecules[5]. In addition, extrinsic factors including myelin proteins such as Nogo, MAG, and OMgp and chondroitin sulphate proteoglycan as a major component of the glial scar in the injured spinal cord prohibit axonal re-growth[6-9]. It was thus suggested that the regulation of these molecules can be a possible target for axonal regeneration in the injured spinal cord[8,10,11].

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Although recent studies increasingly demonstrate successful regeneration of injured spinal cord axons by antibody or gene therapies[11,12], informations on related cellular and molecular events are largely unknown. The injury in an experimental animal model is induced on the spinal cord by transection or contusion. In either case, major cellular responses include inflammatory reactions such as increases in inflammatory cytokines and leukocyte levels[13], and also increased proliferation of non-neuronal cells such as oligodendrocytes and astrocytes. Studies suggested that time-dependent changes in non-neuronal cell proliferation are related to the events including cyst formation, scar formation, or axon sprouting[12,14,15].

In the present study, oriental herbal drug Samul-tang (SMT) was examined as a potential regulatory agent for axonal regeneration in the injured spinal cord. SMT, according to the oriental medicinal treatises, is known to be effective for supplementing hematopoietic function in the bone marrow and hemoglobin function, and thus improve the ischemia[16]. It is also suggested that SMT promotes blood circulation by facilitating vascular activity, and also strengthens the contractility of uterus smooth muscle. Blood-supplementing activity of SMT is further associated with stimulating the function of nervous system, and SMT has been used for treatment of neuronal disorders[17]. We found that SMT is effective for positively regulating molecular factors such as cell

division cycle 2 (Cdc2), axonal growth-associated protein-43 (GAP-43) and external signal regulated protein kinase 1 and 2 (Erk1/2) in the injured spinal cord tissue. SMT also promoted axonal sprouting in the injured corticospinal tract (CST).

Materials and Methods

1. Materials

1) Experimental animals

Sprague-Dawley rats (8 weeks old) were used in this experiment. They were placed in an animal room with regulated temperature (22℃), 60% of humidity, and 12-h light and 12-h dark cycle. They were allowed to eat commercial rat chow and drink water ad libitum.

2) Drugs

Dried Samul-tang (SMT), a combined prescription of Sukjiwhang (Rehmanniae radix preparat), Chungung (Cnidii rhizoma), Danggui (Angelicae gagantis radix), and Baikjakyak (Paeoniae radix alba) was obtained from Daejeon University Oriental Hospital (Daejeon, Korea). Dried drugs were resuspended in 2 liters of water, heat-extracted for 3 h, and filtered three times. The filtered fluid was distilled using the rotary vacuum evaporator (Büchi 461, EYELA, USA). Concentrated solution was frozen at -70℃ for 4 h, and freeze-dried for 24 h. The yield for SMT after freeze-drying was 8 g from 50 g of the initial raw materials. The product was kept at 4℃, and dissolved in water. The stock solution was stored at -20℃ and used for experiment by diluting with physiological saline solution before use.

2. Methods

1) Spinal cord injury procedure

Rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg). Using aseptic technique, a laminectomy was performed to expose dura of the spinal cord at T10 - T11. A contusion injury was induced using a home-made NYU devise by dropping a 10 g weight from 25 mm or 50 mm onto the exposed dura of the spinal cord[18]. The wound was closed and sutured and rats were placed until further treatment in standard plastic cages and given food and water ad libitum.

2) Immunohistochemistry

For immunohistochemistry experiment, dissected tissues were immediately frozen at -75℃ and embedded into the OCT medium. The spinal cord sections (20 μm) were cut using a cryostat and mounted on positively charged slides. Sections were used for immunofluorescence staining, or Hoechst staining. Individual experimental procedures are described below.

(1) Immunofluorescence staining

For immunofluorescence staining, sections on a slide were fixed with 4% paraformaldehyde, 4% sucrose in phosphate-buffered saline (PBS) at room temperature for 40 min, permeablized with 0.5% nonidet P-40 in PBS, and blocked with 2.5% horse serum and 2.5% bovine serum albumin for 4 hr at room temperature. Sections were incubated with anti-GAP-43 antibody (Santa Cruz Biotech, USA), anti-Erk antibody (Santa Cruz Biotech), anti phospho-Erk antibody (Santa Cruz Biotech), anti-Cdc2 antibody (Santa Cruz Biotech), or anti-βIII-tubulin antibody (Covance, USA), then incubated with fluorescein-goat anti-mouse (Molecular probes, USA) or rhodamine-goat anti-rabbit secondary antibodies (Molecular probes) in 2.5% horse serum and 2.5% bovine serum albumin for 1 hr at room temperature and cover-slipped with gelatin mount medium. Primary antibody reaction was performed with single or double antibodies depending on the experimental purposes, and followed with corresponding specific secondary antibody reaction. For some experimental purpose, Hoechst staining reaction for nuclear visualization was performed after the first washing step after secondary antibody reaction (see below for the experimental details of Hoechst staining). Control sections treated with secondary antibody alone usually did not have any visible images. In cases when the nonspecific signals were high, all the data from those experiments were not further analyzed. Sections were observed with a Nikon fluorescence microscope and the images were captured by using Nikon camera. The merged images were produced by using layer blending mode options of the Adobe Photoshop (version 5.5).

(2) Hoechst staining

Hoechst 33258 dye (Sigma, USA) was used to visualize individual cells by staining nucleus. Tissue sections were treated with 25 μg/ml of Hoechst in 0.1% triton X-100 in phosphate-buffered saline solution (PBST) for 10 min. Cell nuclei were observed blue under the fluorescence microscope.

3) Western blot analysis

Nerve tissues were washed with ice-cold PBS, and sonicated under 50 - 200 $\mu\ell$ of triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 25 mM β-glycerophosphate, pH 7.14, 2 mM sodium pyrophosphate, 2 mM EDTA, 1 mM Na_3VO_4, 1% triton X-100, 10% glycerol, 5 mg/ml leupeptin, 5 mg/ml aprotinin, 3 mM benzamidine, 0.5 mM DTT, 1 mM PMSF). Protein (10 μg) was resolved in 12% SDS polyacrylamide gel and transferred to Immobilon polyvinylidenedifluoride (PVDF) membranes (Millipore, USA). Blots were blocked with 5% nonfat dry milk in PBST (17 mM KH_2PO_4, 50 mM Na_2HPO_4, 1.5 mM NaCl, pH 7.4, and 0.05% Tween-20) for 1 hr at room

temperature and then incubated overnight at 4oC in 0.1% triton X-100 in PBS plus 5% nonfat dry milk containing antibodies. Protein bands were detected using Amersham ECL kit (Amersham Pharmacia Biotech, USA), with horseradish peroxidase-conjugated secondary goat anti-rabbit or goat anti-mouse antibodies (Transduction Laboratories, USA). Relative intensities of the protein bands were analyzed by autoradiography. The antibodies used in the present study were anti-Cdc2 antibody, anti-GAP-43 antibody, anti-Erk1/2 antibody, anti-phosphò Erk1/2 antibody, anti-actin antibody (clone no. C4, ICN Biomedicals, USA), and anti-anti-β III-tubulin antibody (Covance, USA).

4) Anterograde and Retrograde tracing

The rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg) and placed in a stereotaxic instrument (Harvard Instrument, USA). Rat's head skin was incised, the skull was drilled, and the glass capillary filled with DiI was lowered through the drilled holes. Labeling of corticospinal tract (CST) was performed by injecting DiI (5 µl of 3% in DMSO) into the sensorimotor cortex [anterior-posterior (AP) 2.0 mm, medial-lateral (ML) 2.3 mm, depth 1.5 mm from the dura surface] on the left side of the brain by using picoinjector (Harvard Instrument, USA), according to the rat brain atlas[19]. DiI diffusion was allowed at least one week and sections of brain and spinal cord at the low thoracic level were prepared and observed under the fluorescence microscope. Retrograde labeling of neuronal cell bodies in the sensorimotor cortex was performed by injecting DiI (5 µl of 3% in DMSO) into the spinal cord at the thoracic level 11-12. Labeling of cortical neuron cell bodies was allowed to diffuse for at least 3 days. To identify labeled cells, animal was anesthetized with ketamine and xylazine and perfused with 4% paraformaldehyde in PBS. Sagittal brain sections were prepared and observed under the fluorescence microscope.

5) Drug administration

Individual herbal drugs (5 µl in PBS) was injected into the crush injury site of the sciatic nerve by using micropipet. For drug treatment into the spinal cord, drug (10 µl) was injected into the contused spinal cord by using micropipet. In case of spinal cord injury experiment, drugs (10 µl) were i.p. supplemented on alternate days for a two week period.

Results

1. Histological analysis of the spinal cord after contusion injury

As an initial step for the use of CST for axonal responsiveness after contusion injury, the spinal cord at thoracic 11 level was exposed and given contusion injury as described in the Materials and Methods. Gravitational impact at both 2.5 cm or 5 cm height resulted in a clear cavity formation in the spinal cord sections (Fig. 1A). Since contusion at 5 cm height impact caused more frequent animal death, contusion impact at 2.5 cm hight was adapted for the rest of the present study. Tissue damage and increased cell numbers were well observed in the impact area at 4 weeks after initial injury (Fig. 1B). A development of cyst, a structure filled with follicles, was also observed in the injury area (Fig. 1C).

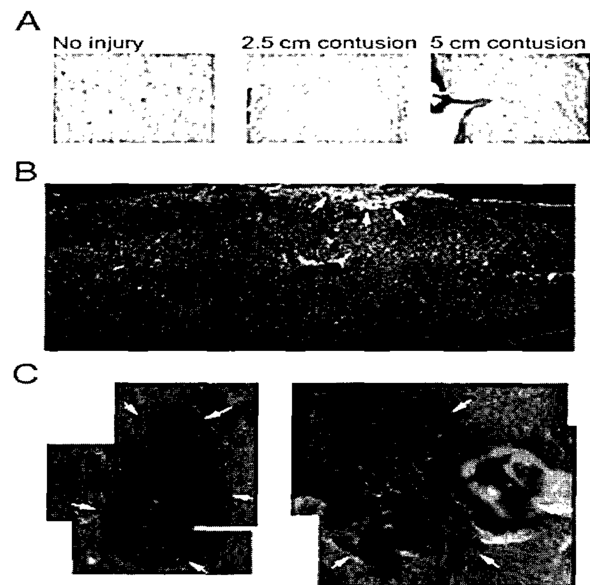

Fig. 1. Histological identification of contusion injury site in the spinal cord. (A) Hemotoxylin and eosin staining in the spinal cord areas with contusion injury with 10 g weight at 2.5 cm or at 5 cm height. (B) Hoechst staining to visualized individual nuclei in the spinal cord sections 2 weeks after contusion injury. (C) Cavity formation in the contusion injury area 4 weeks after impact injury (indicated by arrows).

2. Analysis of GAP-43, Erk1/2, and Cdc2 proteins in the injured spinal cord

Although GAP-43 protein is strictly inducible in the injured peripheral nerves, its regulation in the CNS neurons is variable. As shown in Fig. 2A, Western blot analysis showed constitutive levels of basal expression in the intact nerves. Contusion injury did not change in the injured spinal cord area. SMT treatment slightly up-regulated levels of GAP-43 protein. Immunofluorescence staining showed similar pattern of increased GAP-43 signals in the gray matter area of the transverse sections of the spinal cord after injury with SMT administration (Fig. 2B).

Total Erk1/2 and active form of phospho-Erk1/2 protein levels were investigated in the spinal cord tissue treated with SMT . Basal, similar levels of total Erk1/2 protein were observed with different treatments (Fig. 3A). It was then found that moderate increases in phospho-Erk1/2 protein was observed with SMT treatments when compared with saline-treated control tissue (Fig. 3A). To examine tissue distribution of induced phospho-Erk1/2 protein in the spinal

cord, transverse spinal cord sections were prepared at 10 mm portion caudal to the contusion injury site. Sections from different treatment were used for immunofluorescence staining with anti-phospho-Erk1/2 antibody. Basal levels of phospho-Erk1/2 signal were observed in saline-control as well as non-injury control (Fig. 3B). Clearly increased signals were observed in SMT-treated sections. Double immunofluorescence staining exhibited the presence of overlapping signals in the motor neurons in the ventral horn area (shown in yellow in Fig. 3B). Together with Western results, these data suggest that active form of Erk1/2 protein was increased in the injury area of the spinal cord sections by SMT.

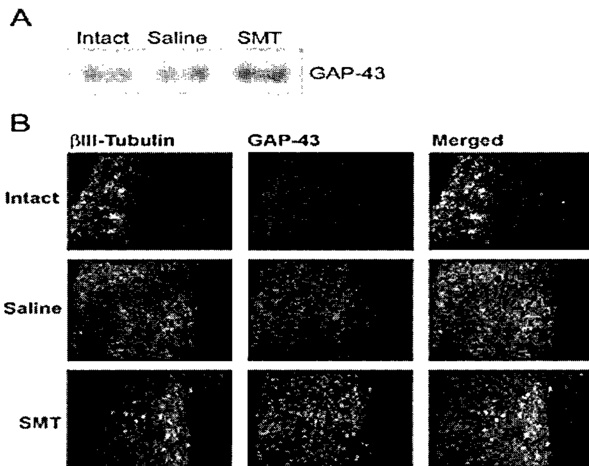

Fig. 2. Induction pattern of GAP-43 protein in the injured spinal cord. Two weeks after contusion injury and drug administration, the spinal cord tissues 1.0 cm rostral and caudal to the injury site were dissected and used for Western and immunofluorescence staining. (A) Western blot analysis of GAP-43 protein. 10 μg of protein extract was used for each lane. (B) Immunofluorescence staining. GAP-43 signal was detected by rhodamine-conjugated secondary antibody and β III-tubulin by fluorescein-conjugated secondary antibody.

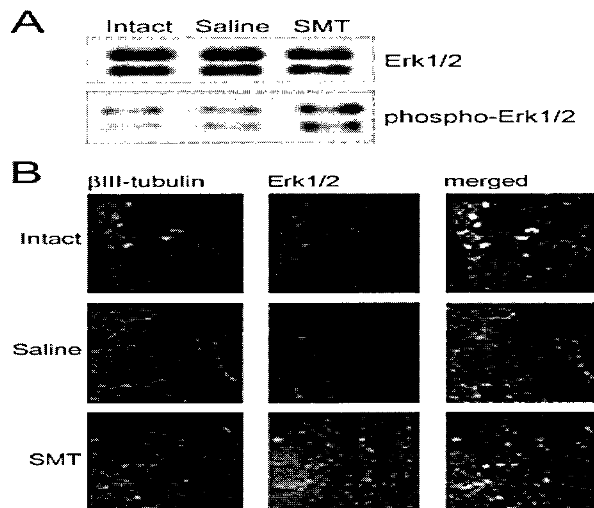

Fig. 3. Induction pattern of Erk1/2 proteins in the spinal cord tissues. (A) Western blot analysis. Two isoforms of Erk1 and 2 were detected as separate bands (42 kD for Erk1 and 44 kD for Erk2). Two weeks after contusion injury, 1 cm length of spinal cord in the injury area tissue was dissected and used for protein extraction. (B). Immunofluorescence staining of spinal cord sections with anti-phospho-Erk1/2 antibody. Two weeks after contusion injury at T11, sections were prepared and transverse sections were used immunofluorescence staining with anti-phospho-Erk1/2 antibody or anti-βIII antibody.

To investigate possible involvement of cell division cycle protein Cdc2 in the injured spinal cord with SMT, protein was prepared from the spinal cord tissue two weeks after injury. Western blot analysis showed low levels of Cdc2 protein in the non-injury control tissue (Fig. 4A). Then, crush injury increased Cdc2 protein in the tissue compared with non-injury control. SMT administration further increased Cdc2 protein levels. In transverse sections at 10 mm caudal to the contusion injury, Cdc2 protein signal was well observed in the gray matter region. Strong signals were found in the spinal cord sections treated with SMT (Fig. 4B). Then, cell types expressing Cdc2 protein was further examined by double immunofluorescence staining. As shown in Fig. 4C, a majority of Cdc2 protein signals was not overlapped with neurons marked by neuron-specific βIII-tubulin signals, suggesting that Cdc2 protein was expressed in non-neuronal cells.

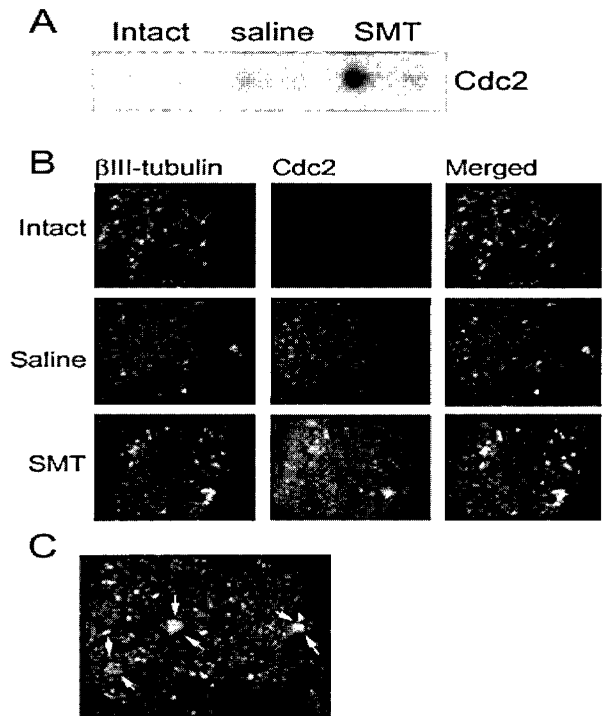

Fig. 4. (A) Western blot analysis of Cdc2 protein in the injured spinal cord tissues along with SMT treatment. Protein was prepared from the spinal cord tissue in the injury area two weeks later. (B). Double immunofluorescence staining of Cdc2 and βIII-tubulin proteins in the transverse spinal cord sections. Comparison of Cdc2 protein signals overlapped with βIII-tubulin was represented in merged images. (C) Cdc2 signals in SMT-treated sections at high resolution image. Cdc2 signals in non-neuronal cells were indicated by arrows.

3. Effects of SMT treatment on non-neuronal cell proliferation

Cdc2 protein is known to play a critical role in the progression of cell cycle. To examine whether increases in Cdc2 protein levels correlated with cell proliferation, changes in cell numbers were measured by counting nuclei stained with Hoechst 33258 dye. As shown in Fig. 5A and B, cell numbers in the injured spinal cord sections were higher in those in

uninjured control sections. SMT treatment further increased cell numbers in the spinal cord sections in the injury area.

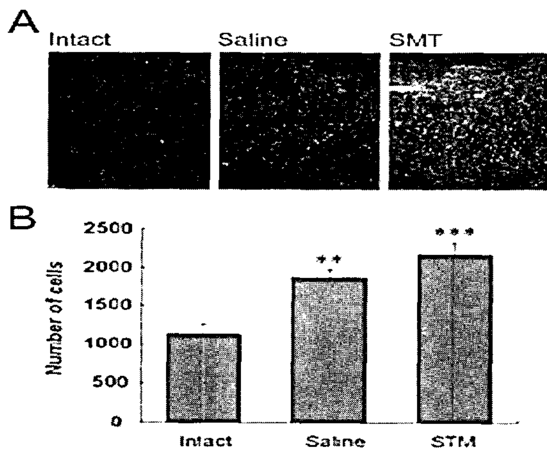

Fig 5. (A) Nuclear staining with Hoechst 33258 dye of transverse sections of spinal cord tissues after different treatments as indicated in the Figure. (B) Cell number determination by counting Hoechst-stained nuclei. **: $p<0.01$, ***: $p<0.001$ compared with intact control group (One-way ANOVA, N=4). Error bars denote standard error of mean (SEM).

4. Effects of SMT on CST axonal paths after contusion injury

The CST begins from the motor cortex in the brain and extends all the way to the lowest part of the spinal cord. For the sake of current experimental identification and utilization, the tract was investigated by retrograde and anterograde tracing techniques. Injections of DiI fluorescein dye into the dorsal half of the spinal cord at the low thoracic levels (T11-12) displayed diffused DiI stains in the motor cortical area of brain 5 days later (retrograde tracing; Fig. 6A,B). Conversely, the injection into the brain coordinate where retrograde signals were heavily detected, similarly showed intense axonal staining by DiI (anterograde labeling; Fig. 6C). Thus, the CST tract was confirmed and histological manipulation for the CST was principally referenced with tracing results for continuing experiment.

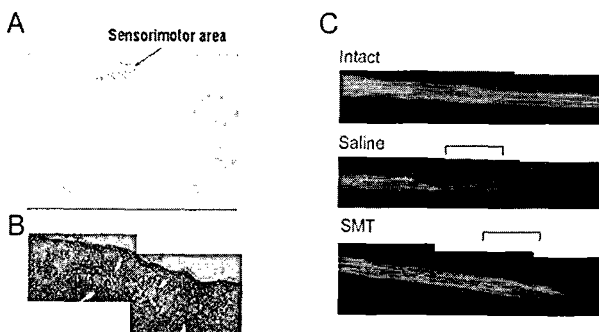

Fig 6. Enhanced axonal elongation of CST in SMT-treated rats. (A) Schematic representation of the motor cortical area in the sagittal section of the rat brain. (B). A montage image of DiI-labeled cortical neurons was merged with neutral red-counterstained brain section. (C). DiI anterograde tracing of CST tracts in the spinal cord at the regions of contusion injury (marked with brackets)

To investigate whether the administration of SMT alters axonal responsiveness after contusion injury, axon tracts were traced by DiI. DiI was injected into the motor cortical area at the time when the contusion injury and drug administration were given at T11 in the spinal cord. Two weeks later, sagittal spinal cord sections were prepared and DiI-labeled axonal tracts were visualized. Increased axonal outgrowth was severely retarded in an epicenter area in the saline-injected spinal cord. Then, administration of SMT improved the extent of axonal sprouting particularly by treatment of CG (Fig. 6C).

Discussion

SMT has been used in the oriental medicine for the treatment of diseases in the cardiovascular and nervous systems[16]. Yet, the actions of SMT at cellular and molecular levels have not been examined in the nervous system. The present study provides a new evidence that SMT is effective for the regulation of neuronal responses in the injured spinal cord.

To investigate possible effects of SMT on axonal regeneration in the CNS, contusion injury model in the rats was established. Contusion model has an advantage above the rhizotomy as a spinal cord injury study because axonal sprouting can be more clearly observed, the extents of inflammatory responses are moderate and the lethality of experimental animals was lower than rhizotomy[20,21]. Furthermore, our data along with previous reports showed that gravitational impact at 2.5 cm with 10 g weight resulted in histologically clear axonal damage in the spinal cord[22-24].

The inability of the CNS environment to support growth appears to be due to both the presence of myelin inhibitory proteins produced from oligodendrocytes or chondroitin sulphate proteoglycans (CSPG) produced from astrocytes and a lack of growth-promoting signals. Previous studies suggest that axonal regeneration could be artificially induced by regulating those inhibitor molecules. Inactivation of inhibitory molecules by knocking out the target genes or administrating antibodies have been reported to induce axonal regeneration and functional recovery[12,25]. It should, however, be noted that most of the studies are currently focused on identifying endogenous molecular factors. In contrast, a realm of the alternative medicine considers a possibility of use of herbal drugs. For instance, herbal drugs have been applied for the induction of peripheral nerve regeneration[26,27]. In the current study, SMT herbal drug was chosen as possible regeneration inducing factors based on a theoretical background of the oriental medicine. SMT, according to the oriental medicinal treatises, can supplement the cardiovascular function particularly by promoting hematopoietic function in the bone marrow and

hemoglobin function. The marrow is classified into bone marrow, brain and spinal cord depending on what they supply. Thus, the nervous system function can be regulated along with bone marrow, and the drugs for cure of marrow problem such as SMT are prescribed for the related diseases including neuronal disorders.

In the spinal cord with contusion injury, we found that SMT treatment up-regulated levels of GAP-43 protein in the injured area compared with saline-treated injury control. GAP-43 has been studied extensively since it was identified in regenerating axons in the peripheral nerves[28,29]. Electrolytic lesions or kainate lesion of hippocampal neurons can increase GAP-43 levels in the affected area, which is associated with increased axonal sprouting[30,31], suggesting axonal growth mediated by GAP-43 in the CNS as well[5]. Western blot analysis revealed increased GAP-43 signals in the motor neurons by SMT treatment. Immunofluorescence staining showed GAP-43 signals in many of non-neuronal cell area as well as neurons, suggesting potential function of this protein for regulation of non-neuronal cells such as astrocytes or oligodendrocytes.

SMT treatment also increased levels of active form of Erk1/2 protein in the injured spinal cord. Erk1/2 is the mitogen activated protein (MAP) kinase consisting of p42 and p44 (Erk2 and 1 respectively) and is the central element in the Ras protein-regulated, mitogenic signal transduction pathway[32]. One of the major consequences of Erk1/2 activation is increased cell proliferation via the activation of cell cycle system. Constitutive activation of Erk1/2 activity in the transfected cells increases the synthesis of Cyclin D1 protein[33]. In this aspect, concomitant increases in Cdc2 protein levels, as has been found in the present study, might play a role in coordinated cell cycle regulation in the non-neuronal cells of the injured spinal cord region. Cdc2 is 34 kDa serine/threonine kinase driving G2 to M phase transition in the cell cycle[34]. Cdc2 is activated by phosphorylation by upstream protein kinase called CAK (Cdk activating kinase) in the presence of activator protein cyclin B1 or B2, and, in turn, activates proteins involved in chromosome condensation, nuclear envelope breakdown, actin cytoskeleton rearrangement, and reorganization of the Golgi apparatus and endoplasmic reticulum. Levels of Cdc2 protein was found to be strongly up-regulated in the injured sciatic nerves and associated with axonal regeneration. Cdc2 protein appears to be highly expressed in the Schwann cells of the regenerating sciatic nerves[35].

In the contusion injured spinal cord, Cdc2 seems to be up-regulated in non-neuronal cells such as astrocytes although immunofluorescence staining data showed that Cdc2 signals were also observed in neuronal cells in the spinal cord. Administration of SMT contributed to prolonged up-regulation of Cdc2 protein levels, suggesting its role for increased cell proliferation. If both Cdc2 and Erk1/2 activities are converged into increasing non-neuronal cell activity, the activity associated with SMT treatment would be antagonistic to the cellular responses to the above-mentioned actions because activated non-neuronal cells could produce inhibitory molecules for axonal regeneration. To a large extent, time-dependent variation of cell proliferation and inflammatory responses after spinal cord injury was reported, indicating that cell proliferation and differentiation of non-neuronal cells in the injury area might have both protective or non-protective roles in relation to the responsiveness after injury[13]. Furthermore, cell-type specific regulation of Cdc2 protein could contribute to differential regulation of injured spinal cord neurons.

As the possible target for changes in axonal responsiveness after contusion injury, we examined the CST using anterograde and retrograde tracing techniques. CST has the cell body in the sensorimotor cortex and extends all the way to the spinal cord where it makes a synaptic contact with motor neurons. Due to a long axonal path, CST is useful for examining axonal responsiveness after injury[2]. In the present study, anterograde tracing was observed 2 weeks after spinal cord injury. Most of proximal axons were terminated around the injury area, and axonal sprouting was not observed well in animal group treated with saline. Administration with SMT increased axonal sprouting in the epicenter, suggesting positive influence of SMT on axonal outgrowth. Previous studies showed axonal sprouting in the injured area when observed longer than a month after the contusion injury[36]. Thus, similar examination for a longer period of contusion injury would be important to further identify possible axonal elongation and compare among animal groups with different treatments.

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