Effects of Oriental Medicinal Drugs on Axonal Regeneration in the Spinal Cord Neurons

Joung-Jo An, Uk Namgung[1], In-Chan Seol, Yoon-Sik Kim*

Department of Internal Medicine, 1: Department of Neurophysiology, College of Oriental Medicine, Daejeon University

An oriental medicinal drugs Jahageo (JHG, Hominis placenta) were examined to determine its effects on the responsiveness of central nervous system neurons after injury. We found that JHG was involved in neurite outgrowth of DRG sensory axons. JHG treatment also increased expression of axonal growth-associated protein GAP-43 in DRG sensory neurons after sciatic nerve injury and in the injured spinal cord. JHG treatment during the spinal cord injury increased induction levels of cell division cycle 2 (Cdc2) protein in DRG as well as in the spinal cord. Histochemical investigation showed that induced Cdc2 in the injured spinal cord was found in non-neuronal cells. These results suggest that JHG regulates activities of non-neuronal cells such as oligodendrocyte and astrocyte in responses to spinal cord injury and protects neuronal responsiveness after axonal damage.

Key words : Hominis placenta, regeneration, spinal cord, DRG sensory neuron, GAP-43, Cdc2

Introduction

The central nervous system (CNS), in contrast to the peripheral nervous system, has limited ability to repair its damaged nerves. When parts of the central nervous system are critically injured, the CNS cannot generate new neurons nor regenerate new axons of previously severed neurons[1-3]. Severed CNS tips initially try to grow, but eventually abort and ultimately completely fail to regenerate[4]. Recently, the search has been growing to identify internal or external molecular factors that will enable axons to regenerate across the injury site and into the injured tissues after damage to the spinal cord. Most fruitful outcomes of these efforts would be the identification of regeneration inhibition factors such as Nogo, Nogo receptor, and myelin-associated glycoprotein (MAG) in the spinal cord[5-11].

Although it is highly conceivable that possible regeneration factors can be sought from exogenous sources such as oriental medicinal herbs, most of the studies on axonal regeneration have been primarily focused on nerve fiber regrowth and elucidating molecular factors, in particular endogenous protein factors. Despite the open possibilities of 'environmental molecular factors' which could be beneficial for axonal regeneration and functional recovery, little is known about their effects on axonal regeneration. Oriental medicinal drugs have been used clinically for the cure of strokes, and brain ischemic damages[12-14]. Yet, specific drugs or drug components in relation to nerve fiber regeneration are not known.

In the present study, oriental medicinal drugs which, according to oriental medicinal theory, are believed to strongly promote kidney have been chosen for their potential effects on injured axon recovery[15]. From the viewpoint of oriental medical theory, 'the kidney is in charge of bones and produces marrow'. The essence stored in the kidney can produce bone marrow which generates the brain, and thus, the growth and functional activities of the brain, bone marrow and skeleton are closely related to the kidney. Brain and spinal cord depend on what they each supply, and sufficient essential energy makes kidney function properly. Therefore, the central and peripheral nerve system can be referred to the marrow, and the drugs promoting kidney are assumed to stimulate the nerve regeneration.

Among drugs which promote kidney, Hominis Placenta (紫河車; JHG) that particularly strengthens muscles and bones[16] has been selected for the present study. We investigated their effects on DRG neurons and spinal cord neurons which were exposed to axonal injury. We found that JHG appeared to be involved in inducing regeneration-related protein factors such as GAP-43 and Cdc2 in DRG and spinal cord, as was effective in the regeneration of peripheral sciatic

* To whom correspondence should be addressed : Yoon-Sik Kim, Department of Internal Medicine, College of Oriental Medicine, DaeJeon University, Youngun-dong, Dong-gu, Daejeon, 300-716, Korea
· E-mail : yoonsik@dju.ac.kr, · Tel : 042-229-6806
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nerves (Jo et al., submitted), Our study also indicates that JHG induced the responsiveness of CNS neurons after nerve fiber injury. The present findings suggest that the oriental medicinal drugs may be involved in regulating regeneration-associated protein factors in the injured spinal cord, and further imply the potential role of oriental medicinal drugs in CNS axonal regeneration.

Materials and Methods

1. Experimental animals

Rats (Sprague-Dawley) were purchased from the local distributer and were fed food pellets (22% crude proteins, 8.0% crude fats, 5.0% crude carbohydrates, 8.0% crude minerals, 0.6% calcium, 0.4% minerals; Samyang Co) and water. The animals were adjusted for 2 weeks at 22±2℃ in a room of a relative humidity of 50±10%, light intensity of 150~300 Lux and 12 hr of electric light (07:00~19:00) and another 12 hr at darkness, and then only healthy animals were selected for experiments.

2. Preparation of oriental medicinal drugs

Hominis Placenta (JHG; 紫河車) was suspended in 2 liter of water, heat-extracted for 3 hr, and filtered three times. The filtered fluid was distilled using the rotary vacuum evaporator. Concentrated solutions were frozen at 70℃ for 4 hr, and freeze-dried for 24 hr. The products were kept at 4℃, and dissolved in PBS. Prepared stock was kept at -20℃ and used for experiment by diluting with physiological saline solution before use.

3. Sciatic nerve and spinal cord surgery

Animals were anesthetized with a mixture of ketamine (80 ㎎/㎏) and xylazine (5 ㎎/㎏). Sciatic nerve was exposed and crushed with a pair of forceps held tightly for 30 sec twice at 1 min intervals[17]. Animals were recovered from anesthesia and sacrificed 3 or 7 days later. Animals were deeply anesthetized with a mixture of ketamine and xylazine, and sciatic nerves, dorsal root ganglion (DRG) or the part of spinal cord were dissected, immediately frozen, and kept at -70℃ until use. For the purpose of immunohistochemistry experiments, the sciatic nerves were prepared by dividing into the proximal stump, 5-㎜ segments proximal to the injury site, and the distal stump, 5-㎜ segments distal to the injury site. For the spinal cord injury, the spinal cord was exposed at lower thoracic level (T11-12) and the dorsal half were cut with microscissors.

4. Histology and immunofluorescence staining

Nerve segments were embedded and frozen at -20℃. Sections (20 ㎛ thickness) were cut on a cryostat and mounted on positively charged slides. Longitudinal or cross sections were prepared. For double immunofluorescence staining, sections were fixed with 4% paraformaldehyde, 4% sucrose in PBS at room temperature for 40 min, permeablilized 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-neurofilament-200 antibody (NF-200, clone no. N52, Sigma), anti-GAP-43 antibody (H-100, Santa Cruz Biotech), anti-βⅢ-tubulin antibody (TUJ1, Covance), anti-S100β antibody (Dako), anti-Cdc2 antibody (p34, Santa Cruz Biotech), then incubated with fluorescein-goat anti-mouse (Molecular probes) and 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. In the present experiment, we always included the control sections treated with secondary antibody alone, which usually did not have any visible images. In cases when the nonspecific signals were high, all the data from that experiment were not further analyzed. Sections were viewed with a Nikon fluorescent microscope and the images were captured by using Axioskop camera. The software Adobe was used to acquire images from the digital camera, and the software Adobe Photoshop (version 5.5) was used to process images. To all the section from the individual experiments, the brightness and contrast of the green and red color images were adjusted essentially to the equal extent when necessary, and the merged images were produced by using layer blending mode options of the Photoshop program.

5. Western blotting

Nerve segments were washed with ice-cold PBS, and sonicated under 50-200 ㎕ 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 ㎍/ml leupeptin, 5 ㎍/㎖ aprotinin, 3 μM benzamidine, 0.5 mM DTT, 1 mM PMSF). Ten micrograms of proteins were used for Western analysis using anti-GAP-43 antibody (H-100, Santa Cruz Biotech.) or anti-Cdc2 antibody (p34, Santa Cruz Biotech). Electrophoresis of membrane proteins was preformed using a 12% SDS-polyacrylamide gel (SDS-PAGE) and then the proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (Pall Corporation, U.S.A). Blocking of nonspecific bindings was done using 3% BSA, 0.1% Tween 20 in TBS buffer for 1 hr at room temperature; then, the membrane was incubated overnight at 4°C. The membrane was washed and then incubated in a solution containing a 1:1000 dilution of polyclonal antibodies

specific for the C-terminal of rat GAP-43 or monoclonal antibodies specific for C-terminal of rat Cdc2 for one and half hour at room temperature. The membrane was washed again and then incubated in a solution containing a 1:1000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Santa Cruz, U.S.A) for one and half hour at room temperature. After washing, blotting proteins were visualized using Western blotting detection system, and then exposed to a Kodak Scientific Imaging Film(Eastman Kodak Co., U.S.A.).

6. Primary DRG sensory neuron culture

Glass coverslips were precoated with poly-L-lysine (overnight at 37℃), and laminin (UBI, Lake Placid, NY; overnight at 4℃) in a 37°C, 5% CO_2 incubator. L4 and L5 ganglia were removed from adult male rats, placed in ice cold Gibco-BRL F14 medium (Life Technologies, Grand Island, NY) containing N1 additives, and rinsed three times in wash medium (F14+N1). The ganglia were placed in F14+N1 containing type XI collagenase for 90 min at 37°C, 5% CO_2. Tissues were then washed three times with wash medium and centrifuged at 800-1000 rpm for 1 min to remove the supernatant. After washing twice with wash medium, cells were dissociated gently with 16-20 passages through a flamed Pasteur pipette, and treated with type SII trypsin followed by trypsin inhibitor (100 ㎍/㎖), and DNase I (80 ㎍/㎖). After washing cells with culture medium, 800-1200 neurons were plated onto 12 round coverslips and cultured for 12 hr and changed with fresh medium. DRG neurons were cultured for 24-36 hr before further analysis.

Results

1. Effects of peripheral or central axon injury on the induction of GAP-43 protein

Rat DRG sensory neurons located in lumber 4-6 have two axonal branches; one connecting to peripheral skin receptors and the other extending to the spinal cord to transmit somatosensory signals. Due to bipolar connections of sensory neuron axons to peripheral and central nervous system, it has been widely used to investigate neuronal responsiveness after axonal injury. To examine whether induction of GAP-43 protein is directly associated with the ability of axonal regeneration of DRG sensory neurons, two sensory axons were separately injured; one in the sciatic nerve and another in the dorsal half of the spinal cord in which mechanosensory neurons ascend to the dorsal column. When the nerve injury was applied to sciatic nerve, induction of GAP-43 protein in the sciatic nerve was detected 3 days after surgery and further increased at 7 days in the sciatic nerve and DRG after surgery (Fig. 1A). However, when dorsal half of spinal cord was cut, any significant changes in GAP-43 protein levels were not detected in DRG and also sciatic nerves. Thus, these data suggest that induction of GAP-43 protein was associated with sensory axonal regeneration in the peripheral, but not central branch.

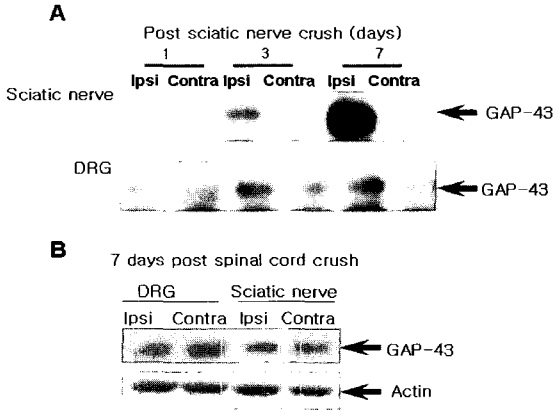

Fig. 1. GAP-43 expression in the DRG after sciatic nerve injury or spinal cord injury. (a) Strong induction of GAP-43 was observed in the sciatic nerves after injury, and weak induction was seen in the DRG. (b) Levels of GAP-43 in the sciatic nerves or DRG after spinal cord injury were not changed above the basal levels. Western blotting with actin was performed as an internal loading control.

2. Effects of JHG treatment on GAP-43 protein levels in the injured spinal cord

Although spinal cord axons cannot regenerate after the injury, recent studies have reported that a partial induction of axonal growth or functional recovery can be achieved by providing induction factors or blocking inhibiting factors. To examine possible involvement of oriental medicinal drugs on spinal cord regeneration, rat spinal cord was exposed at lower thoracic level and dorsal half was cut with microscissors. Levels of GAP-43 protein was investigated by Western analysis and immunofluorescence staining.

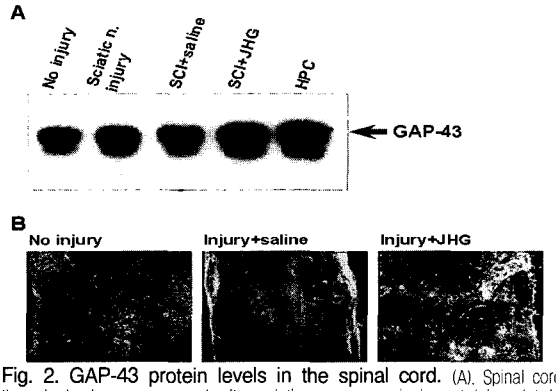

Fig. 2. GAP-43 protein levels in the spinal cord. (A). Spinal cord at lower thoracic levels was prepared after sciatic nerve or spinal cord injury (at lumber 4-5 levels) and induction of GAP-43 protein was analyzed by Western analysis. High levels of GAP-43 protein were similarly observed with in the hippocampus (HPC) tissues. (B). *Immunofluorescence staining. After the excision injury of the dorsal half of* the spinal cord, JHG or equivalent volume of saline was injected into the injury site and intraperitoneally supplemented on a daily basis for 7 days. Spinal cord sections at the injury site were prepared and immunostained with anti-GAP-43 antibodies.

As shown in Figure 2A, GAP-43 protein was increased in an area of injury in the spinal cord, and further increases in DRG from JHG treated animal were observed. Immunofluorescence staining of injured spinal cord showed that induced levels of GAP-43 protein were higher in JHG treated animals compared with saline control group (Fig. 2B).

3. Effect of JHG treatments on neurite outgrowth of DRG sensory neurons

Rat sciatic nerves were crushed and DRG sensory neuron culture was prepared 7 days later. As shown in Fig. 3, neurite outgrowth pattern between no injury and injury group showed clear difference in its length and arborization; marked increases in neurite outgrowth in sciatic nerve crushed animal compared with non-injury control. These data indicate that lesion signals in response to nerve crush induced neurite outgrowth of DRG sensory neurons in culture. Simultaneous injury on the spinal cord along with sciatic nerve crush did not change neurite outgrowth pattern from culture with sciatic nerve crush, suggesting that preconditioning was only effective when the injury was given sciatic, but not spinal cord, nerve which can potentiate axonal regeneration.

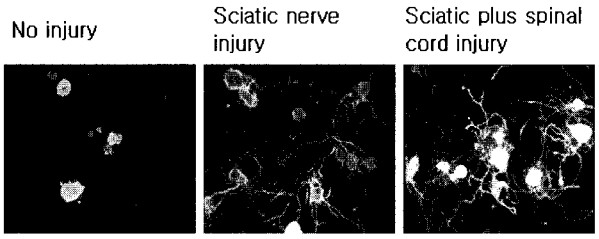

Fig. 3. Preconditioning effects on neurite outgrowth on cultured DRG sensory neurons. One week after sciatic nerve or spinal cord injury, primary DRG sensory neurons were prepared and cultured for 36 hr. Pattern of neurite outgrowth was examined by staining cultured neurons with neuron-specific tubulin antibody. Neurons pretreated with sciatic nerve injury or sciatic nerve plus spinal cord injuries showed more branched and elongated neurite outgrowth.

Using this experimental paradigm, possible effects of JHG on axonal regeneration of DRG sensory neurons in terms of neurite outgrowth were investigated. DRG sensory neurons were cultured for 30 hr, and then treated with JHG (50 $\mu g/m\ell$ or 200 $\mu g/m\ell$). Cells were collected 24 hr later and fixed for immunofluorescence staining with tubulin and GAP-43 antibodies. βIII-tubulin subtype analyzed in the present study was neuron-specific, which is abundant in axons and thus used to visualize neurite extension. Axonal growth associated protein with an apparent molecular weight 43 KDa (GAP-43) is highly induced in neurons after nerve injury. Therefore, these two proteins were used to determine neurite outgrowth pattern in culture DRG sensory neurons. Neurons treated with 50 $\mu g/m\ell$ of JHG showed more elongation of neurite outgrowth compared with saline treated group (shown by Tubulin immunostaining of cultured DRG neurons in Fig. 4). When JHG concentration was increased to 200 $\mu g/m\ell$, neurite growth was reduced compared with 50 $\mu g/m\ell$ JHG treated group, but higher than saline control group. These results suggest that JHG treatment might play a role in inducing neurite outgrowth of preconditioned cultured DRG sensory neurons.

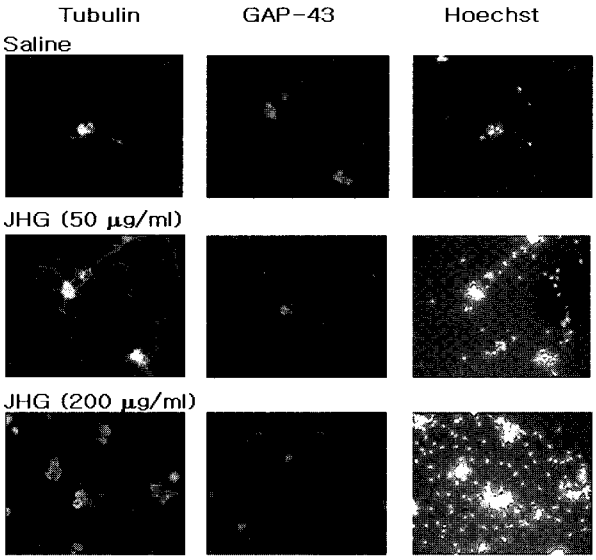

Fig. 4. Effects of JHG treatment on neurite outgrowth and GAP-43 protein levels in cultured DRG neurons. DRG sensory neurons after sciatic nerve injury preconditioning were cultured for 24 hr in the presence of 50 $\mu g/ml$ or 200 $\mu g/ml$ JHG or saline (vehicle control). Tubulin staining showed enhanced neurite outgrowth in cells treated with 50 $\mu g/ml$ JHG. GAP-43 levels were also increased in this cell group compared to saline control or 200 $\mu g/ml$ of JHG treated cells. Individual cell nuclei were visualized by Hoechst staining.

4. Effects of oriental herbal drugs on the induction of Cdc2 protein in DRG

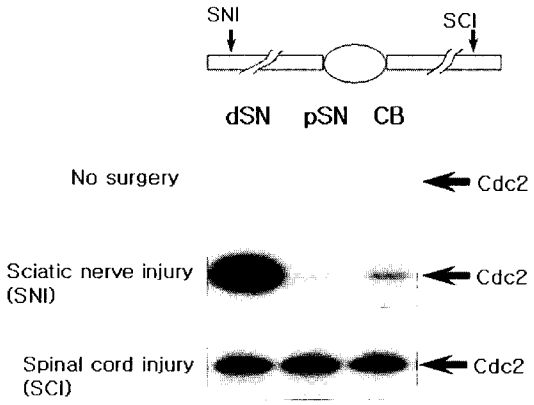

Fig. 5. Cdc2 induction in the DRG sensory neuron system after nerve injury. The diagram represents injury sites- one on the peripheral axon branch sciatic nerve (SNI: sciatic nerve injury) and another on the central branch on the spinal cord (SCI: sciatic cord branch). Cell lysates were prepared from dorsal sciatic nerve (dSN), proximal sciatic nerve close to the DRG (pSN), and cell body (CB) in the DRG. Cdc2 protein levels from different treatments were analyzed by Western blotting with anti-Cdc2 antibody.

Cell division cycle 2 (Cdc2) is primarily important for cell cycle progression in proliferating cells. Yet, little is known about its function in postmitotic cells such as neurons. In the

sciatic nerves and DRG with no injury stimulation, no Cdc2 protein was detected. Then with sciatic nerve injury, strong induction of Cdc2 protein was detected in the injured sciatic nerve and DRG tissue (Fig. 5). When the dorsal half of the spinal cord at the lower thoracic levels was severed, Cdc2 protein was also strongly induced in the sciatic nerve and DRG cell body.

To study whether JHG affects on induction levels of Cdc2 protein in neuron in the CNS environment, neurons in DRG after sciatic nerve injury were investigated by immunofluorescence staining. Cdc2 protein was increased in the DRG after sciatic nerve injury, and further increases were observed by JHG treatment during nerve injury (Fig. 6). Cdc2 kinase activator protein cyclin B1 did not show any significant changes in its level by injury and JHG treatment. To localize Cdc2 expression in DRG, the same section used for Cdc2 immunostaining was also immunoreacted with S100β protein which is selectively expressed in Schwann cells (Fig. 7). It was observed that a large portion of Cdc2 signal was overlapped with S100β signal, suggesting Cdc2 expression in Schwann cells.

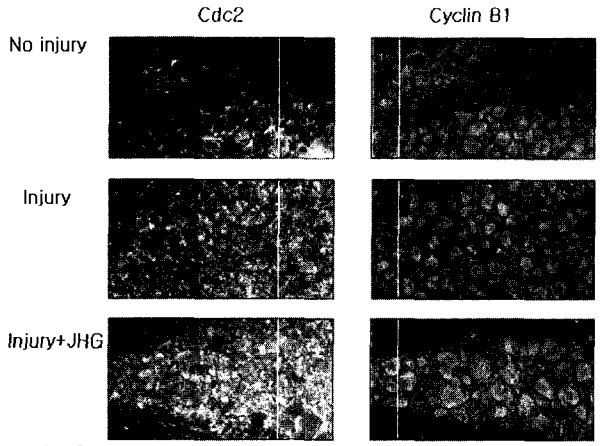

Fig. 6. Cdc2 and cyclin B1 protein levels in the DRG after sciatic nerve injury. Seven days after sciatic nerve injury and also with JHG treatment, DRG sections were prepared for immunofluorescence staining with anti-Cdc2 antibody or with cyclin B1 for individual sections. Cdc2 protein and cyclin B1 signals were mostly overlapped in the same locations.

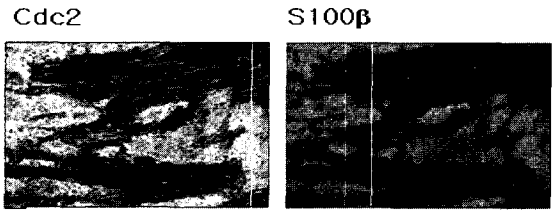

Fig. 7. Localization of Cdc2 protein signals in the DRG sections. After sciatic nerve injury and JHG treatment, DRG sections were prepared and used for immunostaining with both Cdc2 and S100β antibodies.

5. Effects of JHG treatment on Cdc2 protein levels in the injured spinal cord

In no injury control, Cdc2 protein levels were very low, but small increases were found after sciatic nerve injury (Fig. 8). Interestingly, strong induction of Cdc2 was found after spinal cord injury. To determine which spinal cord tissues were responsible for Cdc2 increase, spinal cord sections at the injury site were used for immunofluorescence staining with Cdc2 antibody. As shown in Fig. 9A, induced Cdc2 protein was largely colocalized with the expression area of S100β, a non-neuronal cell marker protein. Injured spinal cord along with JHG treatment showed further increases in Cdc2 protein levels in the spinal cord. Increased Cdc2 protein was associated with increased numbers of Hoechst stained nuclei of non-neuronal cells in the injury area (Fig. 9B). Together, these results suggest that increased Cdc2 expression might be associated with increased proliferation of non-neuronal cells in the injury area.

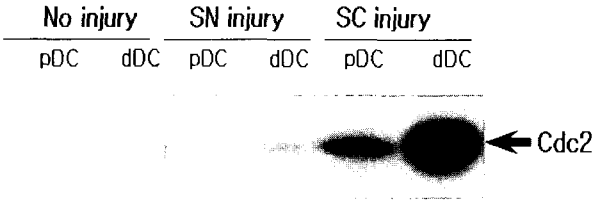

Fig. 8. Western blot analysis of Cdc2 protein in the spinal cord after spinal cord injury. Spinal cord samples were prepared and cell lysates of the spinal cord were used for Western blotting for Cdc2 protein measurement. pDC represents the central branch of the DRG sensory neurons close to the DRG, and DC represents the dorsal column at the lower thoracic level where the spinal cord injury (SCI) was given. SN injury: sciatic nerve injury, SC injury: spinal cord injury.

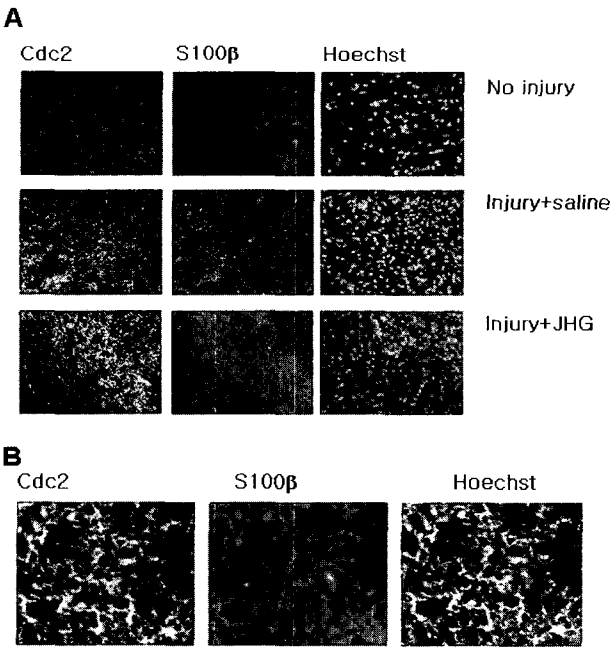

Fig. 9. Cdc2 protein expression in the injured spinal cord. After the excision injury of the dorsal half of the spinal cord, JHG or equivalent volume of saline was injected into the injury site and additionally administrated intraperitoneally on a daily basis (0.5 ml of 100 mg/ml drug in 0.9% saline). (A) Spinal cord section at the injury site was prepared and immunostained with anti-Cdc2 or anti S100β antibodies. Individual cells were also visualized by Hoechst nuclear staining. (B) The three images at the lowest panel are the enlarged views of the sample with injury plus JHG treatment and demonstrate that Cdc2 protein and S100β signaling were partly overlapped (seen in yellow under fluorescence microscope), but other areas were seen only with Cdc2, implying the possibility of Cdc2 protein induction in neuronal tissues.

Discussion

In view of the anatomical arrangement of its ascending and descending fiber tracts, even local limited injury to the spinal cord typically has devastating consequences[2]. Accordingly, much effort has been focused on characterizing endogenous molecular factors which may regulate axonal regeneration after injury. A few of studies have indicated that oriental herbal drugs might play some role in promoting axonal regeneration. For instance, Gorio et al.[18,19] have reported that ganglioside, one of the major ginseng components improved sciatic nerve recovery after injury and neuronal functional recovery. Also, Cheng et al.[20] reported that buyang huanwu decoction was effective on inducing sciatic nerve regeneration. Furthermore, other studies implicated that oriental herbal drugs can improve neural functions, for instance by facilitating the recovery of neurons in an area of ischemic injury, or helping rehabilitating ability after various kinds of nervous system-associated disorders. Despite these implications, experimental evidences based on quantitative measurements are very scarce. Thus, we have investigated the potential role of oriental medicinal drugs on spinal cord regeneration. The crucial step for this early study would be to select target drugs and examine whether they have any function on axonal regeneration process. Molecular factors effective for the induction of axonal regeneration in the peripheral nervous system are capable of regenerating of CNS neurons. In a separate study performed by our group, we examined 8 different herbal drugs and identified three (Jahageo, Golsebo, and Baikhasuo) drugs as being effective for sciatic nerve regeneration. These results prompted us to examine JHG, the most prominent in relation to CNS axon regeneration.

We have utilized the advantages of DRG neuron system to investigate the potential application of oriental medicinal drugs toward CNS axon regeneration[21,22]. We found first that DRG sensory neurons of preconditioned rats showed enhanced neurite outgrowth promoting activity compared with non-injury control group. Then, the treatment of JHG in the cultured neurons further induced neurite outgrowth, suggesting that JHG has a similar function as lesion signals for axonal regeneration. Moreover, GAP-43 protein levels, known to be an important determinant for axonal regeneration after injury[23] in DRG sensory neurons was increased by JHG treatment, suggesting that JHG may have a growth promoting role for regenerating axons. Thus, growth promoting activity induced from peripheral axon stimulation can induce sensory axon regeneration located in the spinal cord, implying that JHG could induce spinal cord axon regeneration after injury.

Cdc2 is a kinase which functions in the progression of cell cycle by facilitating G2 into M phase shift[24,25]. In the present study, a role of Cdc2 in axonal regeneration was examined, and the results showed that Cdc2 protein was induced in a complicated manner. Cdc2 protein was strongly induced in the injured sciatic nerves or in the injured spinal cord. In the DRG, a slight Cdc2 induction was observed after sciatic nerve injury whereas it was strongly induced by spinal cord injury. Immunofluorescence staining showed induced Cdc2 signals overlapping with S100β signal, a non-neuronal marker protein, suggesting that a majority of Cdc2 proteins was induced in the non-neuronal cells (for example in the Schwann cells in the sciatic nerve and oligodendrocytes in the spinal cord). In the DRG, Cdc2 protein was induced in non-neuronal Schwann cells as well. One possible interpretation of these data is that Cdc2 might be involved in axonal regeneration by promoting non-neuronal cell proliferation both in CNS and PNS. After injury, rapid proliferation of non-neuronal cells are well observed in an injury area, which could be important for axonal guidance. We speculate that Cdc2 protein kinase activity induced in the non-neuronal cells such as in astrocytes and oligodendrocytes increases cell proliferation, which is then functionally involved in protecting neuronal cells undergoing axonal injury by regulating inflammation or the production of molecular factors[7,8,26,27](e.g., Nogo, MAG, chondroitin sulphate proteoglycan).

In summary, we have found that oriental medicinal drug JHG can regulate the responsiveness of injured CNS neurons possibly in favor of rehabilitating neuronal activity. Combined use with other drugs such as ginsenoside which was reported to be effective for inducing peripheral nerve regeneration[28] may improve its effects on axonal recovery from spinal cord injury.

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