Enhanced Neurite Outgrowth of Dorsal Root Ganglion Sensory Neurons after Sibjeondaebo-tang Treatment

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Sibjeondaebo-tang (SJDBT) is an oriental medicinal prescription for the treatments of diverse symptoms including neurological disorders. In order to investigate its potential role for neural regulation following nerve injury, neurite outgrowth of dorsal root ganglion (DRG) neurons in culture was investigated. In DRG neurons which were preconditioned by sciatic nerve injury, neurite outgrowth was enhanced by SJDBT treatment. When preconditioned DRG neurons were co-cultured with astrocytes prepared from injured spinal cord tissue, neurite outgrowth was similarly facilitated by SJDBT. Astrocytes in co-culture showed more intense signals of vimentin protein by SJDBT compared to saline control. Sukjihwang (SJH), a conventional herbal component of SJDBT prescription, did not induce any significant changes in neurite extension of DRG neurons compared to control cells. These data suggest that SJDBT may be the therapeutic agent for nervous system disorders related to nerve damage.

Key words : Sibjeondaebo-tang (SJDBT), dorsal root ganglion (DRG), neurofilament-200 (NF-200), vimentin, co-culture

Introduction

Sibjeondaebo-tang (SJDBT) is the prescription used in an oriental medicine for replenishing circulatory system and 'qi' (energy flow), which is particularly useful to treat Soeum person who is believed to show weak blood flow and weak 'qi'[1]. Several experimental studies have reported that SJDBT has an anti-oxidant activity and is related to anti-allergic immune responses as well as anti-tumor effects[2-5]. Furthermore, SJDBT treatment protected neurons from oxidative stress or from glutamate-induced toxicity[6-8], suggesting potential role of SJDBT in neuroprotection. However, whether SJDBT treatment is further associated with neurotrophic activity remains to be studied.

Sensory neurons have a bifurcated axon structure forming peripheral and spinal nerves, and are primarily responsible for transmitting somatosensory signals such as pain, touch, and proprioceptions. In general, axons of sensory neurons can regenerate when the axonal injury is given to the peripheral branch, but cannot regenerate by the injury on the spinal nerve branch, which reflects the difference of environmental influences between peripheral and central nervous systems. Unlike other neurons in the central nervous system, dorsal root ganglion (DRG) neurons isolated from adult animals can be cultivated for several days, and thus have been used as a useful tool for in vitro investigation of axonal regeneration. For instance, rat DRG sensory neurons at lumbar 4 and 5 show enhanced neurite outgrowth when the neurons are 'preconditioned' by sciatic nerve injury for several days[9]. Thus, the extent of neurite outgrowth of cultured DRG neurons can be used as an in vitro indicator for axonal regeneration[10].

It has been recently shown that several oriental medicinal therapies such as Sengmaek-san (Shengmai-san), ginsenoside, and Jahageo (*Hominis placenta*) are effective for inducing regenerative responses after nerve damage in both peripheral nerves and spinal cord[11-13]. In the present study, we investigated potential effects of SJDBT on neurite outgrowth of cultured DRG sensory neurons, together with SJH, one of the conventional herbal components of SJDBT for comparative analysis. To further examine the effects of environmental factors in the spinal cord, DRG sensory neurons were co-cultured with astrocytes prepared from spinal cord tissue after injury. Our data showed that SJDBT is effective for increasing neurite outgrowth of DRG sensory neurons in single culture or co-culture system, implicating that SJDBT may have growth-promoting activity on injured nerves.

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Materials and Methods

1. Experimental animals and anesthesia

Sprague-Dawley rats (male, 8 weeks old, 200-250 g; Daehan Biolink Inc, Eumseong, Korea) were maintained in an animal room with regulated temperature (20±2℃), 50±5% of humidity, and 12-hr light/dark cycle (lights on 0700 hr to 1900 hr). They were allowed to eat commercial rat pellet chows (Cargill Agri Purina, Inc., Seongnam, Korea) and drink water *ad libitum*. The animals were retrieved from the cage and placed in a tared beaker and determined its weight. After manually restraining the animals, rats were deeply anesthetized by injecting intraperitoneally a single dose of a mixture of ketamine-hydrochloride (80 mg/kg, Yuhanyangheng, Inc., Seoul, Korea) and xylazine-hydrochloride (5 mg/kg, 2% injection, Bayer Korea, Ltd., Ansan, Korea). Usage of ketamine-hydrochloride for academical purpose was approved by Daejeon office of Korea Food and Drug Administration.

2. SJDBT and SJH preparation

Dried SJDBT, which consists of ten herbal components with current clinical modification (Table 1) was obtained from Oriental Medical Hospital of Daejeon University (Daejeon, Korea). The extract was frozen at -75℃ (Nuaire ultralow laboratory freezer, Plymouth, MN, USA) for 4 h and freeze-dried for 24 h. The yields of SJDBT and SJH were 14.8 g for 50.5 g and 8 g for 50 g from the initial raw materials. Purified materials were stored at -75℃ and used for the experiments after dissolving them in physiological saline solution (1 mg of extract residue/ml in 0.9% NaCl solution).

Table 1. Prescription of Sibjeondaebo-tang (SJDBT)

Herb	Galenical name	Amount (g)
人 蔘	*Ginseng Radix*	3.75
白何首烏	*Cynanchi Wilfordii Radix*	3.75
官 桂	*Cinnamomi Cortex Interior*	3.75
黃 芪	*Astragali Radix*	3.75
白 朮	*Atractylodis Macrocephalae Rhizoma*	3.75
當 歸	*Angelicae Gigantis Radix*	3.75
川 芎	*Ligustici Rhizoma*	3.75
白芍藥	*Paeoniae Radix Alba*	3.75
陳 皮	*Citri Pericarpium*	3.75
炙甘草	*Glycyrrhizae Radix*	3.75
生 薑	*Zingiberis Rhizoma Recens*	3.75
大 棗	*Zizyphi Fructus*	3.75
	Total amount	45.00

3. Sciatic nerve and spinal cord surgery

Sciatic nerves on both hind limbs were exposed at the mid-thigh level through the space between the pelvic head and vertebral head of biceps femoris muscle on the lateral side of the thigh. The nerves were crushed with a pair of forceps held tightly for 30 sec twice at 1 min intervals.

Using aseptic technique, a laminectomy was performed to expose dura mater of the spinal cord at thoracic levels of 9 - 10. A contusion injury was given to the exposed dura using the NYU compactor by dropping a 10 g impactor (a cylindrical metal rod tapered to a tip diameter of 2 mm) from 2.5 cm height. After the suture of the wound in anatomical layers, rats were maintained in standard plastic cages until further treatment. Rat care and all experimental procedures were in accordance with the Animal-use Statement and Ethics Committee approval statement for animal experiments at Daejeon University. A total of 23 rats were used in this study.

4. DRG sensory neuron culture and DRG-Astrocyte co-culture

For DRG neuron culture, DRG at lumbar 4 and 5 of the intact rat or rats with sciatic nerve injury (3 days) were dissociated by treatment with 125 U/ml type XI collagenase (Sigma, St. Louis, MO, USA) in BME (Basal Medium Eagle, Sigma) for 90 min at 37℃(CO_2 incubator; Revco Elite II; Thermo scientific, Waltham, USA) and washed twice with BME. Cells were treated with 0.5 mg/ml type SII trypsin for 15 min, followed by an inhibition reaction for 5 min in 1 mM EDTA, 100 μg/ml of soybean trypsin inhibitor, and 40 μg/ml of DNase I. Cells (1×10^5 cells) were plated onto 12-mm coverslips (Bellco Glass Inc., Vineland, NJ, USA) precoated with 0.01% poly-L-ornithine (Sigma) and laminin (0.02 mg/ml; Collaborative Research, Bedford, MA, USA) in BME containing 5% fetal bovine serum (GIBCO, Melbourne, Australia) plus 5% horse serum, 2 mM glutamine, and 1% penicillin-streptomycin (BME with serums). After 3 hr of the plating, medium was changed with the same BME with serums. Eight hours later, cells were treated with 0.6 mg/ml of SJDBT, 0.6 mg/ml SJH, or equivalent volume of saline vehicle and incubated for 48 h.

For co-culture of DRG sensory neurons with spinal cord non-neuronal cells, a dorsal half of spinal cord covering 0.5-cm length of the rostral and caudal region from the injury site was dissected from rats that had given spinal cord injury for 3 days. Spinal cord non-neuronal cells (1×10^5 cells per 12-mm coverslip) were incubated for 24 h before the addition of freshly prepared DRG sensory neurons (1×10^5 cells), which were prepared from rats that had undergone sciatic nerve injury for 3 days ('preconditioned'). Co-cultured cells were treated for 48 h with SJDBT and SJH (0.6 mg/ml each) or saline vehicle. Dose-response neurite outgrowth of DRG neurons was determined in a range of 0.2 - 2.0 mg/ml of SJDBT or SJH in culture, and the doses of 0.6 mg/ml of SJDBT

and 0.6 mg/ml of SJH that resulted in the highest neurite outgrowth were used for the present study.

5. Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde at room temperature for 45 min, permeabilized with 0.5% nonidet P-40 in PBS (Phosphate buffered saline), and blocked with 2.5% horse serum and 2.5% bovine serum albumin for 4 hours at room temperature.

DRG sensory neurons and co-cultured non-neuronal cells were subjected to immunofluorescence staining with primary antibodies against to NF-200 (neurofilament-200, rabbit polyclonal, 1:400; Sigma) and anti-vimentin proteins (mouse monoclonal, 1:1000; Cell Signaling Technology, Danvers, MA, USA) respectively, and detected by rhodamine-labeled goat anti-rabbit IgG (1:400; Molecular Probes, USA) or fluorescein-labeled goat anti-mouse IgG (1:400; Molecular Probes) secondary antibody in 2.5% horse serum and 2.5% bovine serum albumin for 1 hour at room temperature. Cellular nuclei were stained with 2.5 μg/ml of Hoechst 33258 (bis-benzimide, Sigma) for 10 minutes before the final washing with 0.1% Triton X-100 in PBS, and the cells were mounted with gelatin mount medium (Glycerol and gelatin; Sigma).

6. Image processing and measurement of neurite length of DRG sensory neurons

Cultured cells in 24-well plate samples were viewed at 12 hr and 60 hr time points with a phase contrast inverted microscope (Nikon Eclipse TE2000-U) equipped with a digital camera (Nikon DS-U2). Labeled cells on the coverslip were viewed with a fluorescence microscope (Nikon Eclipse E-600; Nikon, Tokyo, Japan) equipped with a digital camera (Nikon DXM 1200F, Nikon). Digital images were captured and transferred to the Adobe Photoshop program (version 7.0, Korea Adobe Systems, Inc., Seoul, Korea). The length of neurite processes exhibiting clear outgrowth (longer than cell body size) were analyzed by the i-Solution software program (Image and Microscope Technology, Goleta, CA, USA). Mean neurite length was determined by analyzing at least 20 sensory neurons grown on the coverslip.

7. Statistical analysis

Data were presented as the mean± standard error of mean (S.E.M.). The mean values in individual groups were compared by one-way analysis of variance (ANOVA) with Tukey's post hoc test (SPSS version 14.0; SPSS Korea Data Solutions, Inc., Seoul, Korea), and statistically significant difference was reported as $p<0.05$.

Results

To determine the effects of SJDBT on morphological features of DRG sensory neurons in culture, DRG at lumbar 4 and 5 levels was isolated from rats in which preconditioning sciatic nerve injury had or had not given for 3 days. Measurement of neurite outgrowth of DRG neurons after 60 hr culture showed significantly increased neurite outgrowth in preconditioned group compared to untreated control group (234±17 ㎛ vs. 77±10 ㎛, $p< 0.001$, Fig. 1A and B). Neurite length of preconditioned neurons was further increased by SJDBT treatment (234±17 vs. 338±13 ㎛, $p<0.01$). However, SJH treatment did not induce any significant changes in neurite elongation.

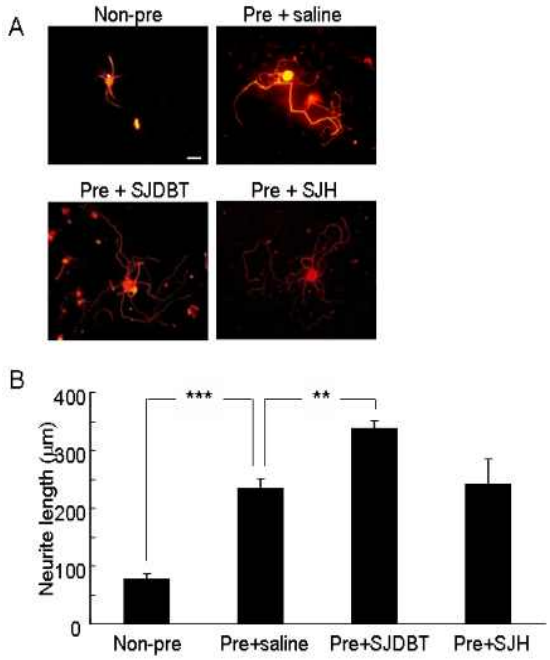

Fig. 1. SJDBT-mediated enhancement of neurite outgrowth of DRG sensory neurons. Prior to DRG preparation, animals had or had not been subjected to preconditioning crush injury in the sciatic nerve for 3 days (labeled 'Pre' or 'Non-pre' in the figure). DRG neurons were cultured for 60 hr and harvested for immunofluorescence staining with anti-NF-200 antibody. In preconditioned cells, either SJDBT (0.6 mg/ml), SJH (0.6 mg/ml) or equivalent volume of saline was treated for 48 hr. (A) Representative immunofluorescence view of NF-200-labeled DRG neurons. (B) Comparison of mean neurite length among different groups. Statistical comparison between preconditioned cells with saline (Pre+saline) and SJH treatments (Pre+SJH) showed no significant difference. (***P<0.001, **P<0.01, N=6, one-way ANOVA). Scale bars = 50 ㎛.

To further examine whether SJDBT affects on DRG neuronal morphology, preconditioned cells were observed by phase-contrast microscope at 12 hr or 60 hr time point after the beginning of the culture. After 12 hr culture, both saline- and SJDBT-treated cells began to initiate neurite growth processes, but did not show remarkable differences in neurite length, although larger sizes of cell bodies were observed more

frequently in SJDBT-treated group (see enlarged image in Fig. 2A). When cells were cultured for 60 hr, more elongated neurites with a swollen shape of cell body were observed more frequently in SJDBT-treated group compared to saline control.

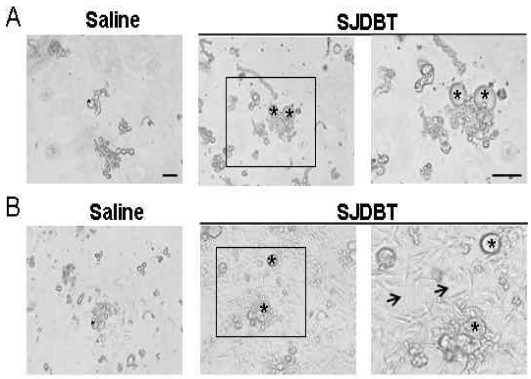

Fig. 2. Enhanced growth processes of DRG sensory neurons by SJDBT treatment. Preconditioned DRG neurons were observed by phase contrast microscope at 12 hr (A) or 60 hr (B) time points after culture. In SJDBT-treated cells, newly emerging growth processes from round-shape cell bodies (marked asterisks) were clearly seen at 12 hr time point. Notice that at 60 hr culture, growth processes are observed with higher density in SJDBT-treated group compared to saline control (arrows). Scale bars in (A) = 50 ㎛, which also apply to all images in (B).

To investigate possible effects of SJDBT on neurite outgrowth of DRG neurons under CNS environment, DRG neurons were co-cultured with primary non-neuronal cells of rat spinal cord which had been prepared 3 days after spinal cord injury. DRG sensory neurons in co-culture showed moderate levels of neurite outgrowth. SJDBT treatment significantly improved neurite elongation compared to saline control (330±25 ㎛ vs 222±16 ㎛, $p<0.001$). However, mean neurite length was similar between SJH- and saline-treated groups (236±5 ㎛ vs 223±16 ㎛, $p = 0.4$)(Fig. 3).

Since DRG neurons were cultivated with spinal cord non-neuronal cells, enhanced neurite outgrowth by SJDBT could be mediated by either DRG neurons or co-cultured non-neuronal cells. Double immunofluorescence staining of cultured non-neuronal cells revealed that 98% of Hoechst 33258-stained nuclei in the culture field were positive to astrocyte marker protein GFAP, and 94% of GFAP-positive cells were positive to vimentin immunostaining. In co-culture system, vimentin-labeled cells were clearly identified(Fig. 4), and in cells treated with SJDBT, intensity of vimentin signal was stronger than saline and SJH-treated cells. Moreover, the shapes of DRG neuron cell body were larger and more round than saline control or cells treated with SJH. In SJH-treated cells, aggregated pattern of vimentin-labeled cells was observed whereas corresponding cells in SJDBT-treated group were distinctively separated each other and prone to form a cluster adjoining to the neurite processes.

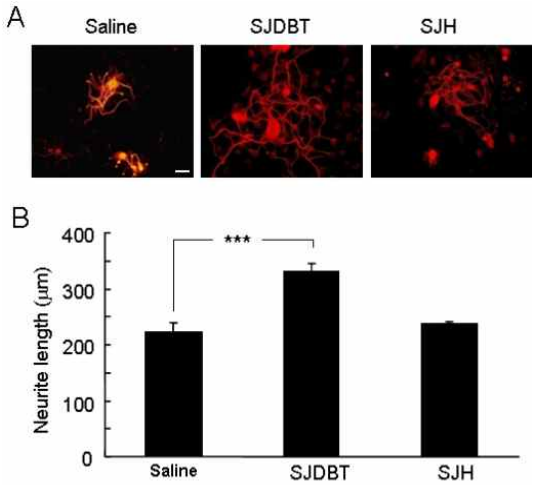

Fig. 3. Enhanced neurite outgrowth by SJDBT in DRG neurons co-cultured with spinal cord non-neuronal cells. Spinal cord cells were cultured for 24 hr prior to DRG neuron addition. Cells were further cultured 48 hr in the presence of SJDBT, SJH or saline. (A) Representative fluorescence views of NF-200-labeled sensory neurons. (B) Comparison of neurite length of DRG sensory neurons after SJDBT, SJH, or saline treatments. SJDBT treatment significantly increased neurite length compared to saline treated cells (***$P<0.001$, $N=6$, one-way ANOVA). Comparison of mean neurite length between saline and SJH treatment did not show any significant difference. Scale bar = 50 ㎛.

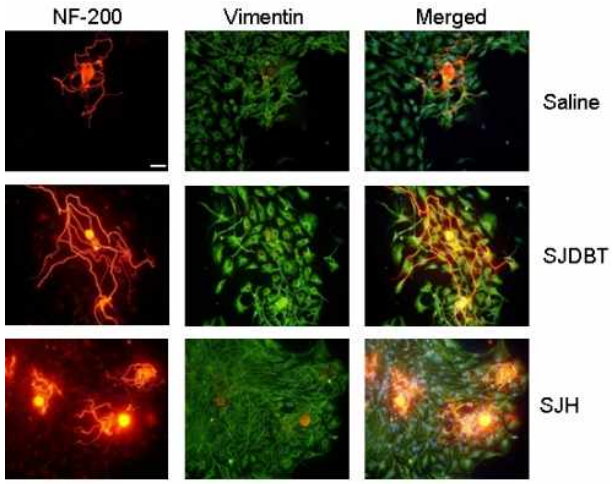

Fig. 4. Increased vimentin staining of spinal cord non-neuronal cells by SJDBT. Co-cultured cells were subjected to double immunofluorescence staining with NF-200 (green) and vimentin to detect DRG neurons and non-neuronal cells (red). Most intense and clear staining for non-neuronal cells was observed in a group treated with SJDBT. Scale bars = 50 ㎛

Discussion

In Korean traditional medicine, SJDBT prescription has been used to reinforce the healthy energy flow to individuals who are depleted of qi because of the pathogenic conditions or chronic diseases[14]. Due to the nature that potential efficacy of SJDBT is descriptive without quantitative evidence, it is hard to define specifically the effects of SJDBT on the

pathophysiological basis. However, several experimental studies suggest that SJDBT has protective function in cells or tissues and helps maintain immune activity by properly regulating macrophages, T lymphocytes, and natural killer cells. It has been reported in nervous system that SJDBT treatment can improve cholinergic neuronal activity that could be related to senile dementia and thus cerebral energy generation[8]. As an initial step to examine possible regulatory activity of SJDBT on nerve regeneration, we have begun to determine changes in neurite outgrowth of DRG sensory neurons. Besides SJDBT, effects of SJH, one of the conventional herbal components of SJDBT, on neurite outgrowth were analyzed together.

DRG sensory neurons show bifurcating axon structures that are extended both to peripheral sensory target and spinal cord neurons, and thus have been used to investigate regenerative responses of axonal injuries given to peripheral or spinal nerves. For instance, axonal regeneration does not occur when the nerve injury is given to spinal branch nerve, but does occur with nerve injuries at the spinal branch[15]. When the nerve injury was given to both peripheral and spinal branches, axonal regeneration can be induced at the spinal cord in addition to peripheral axons. Since lesion signals induced at the peripheral nerve can be retrogradely transported into the cell body and lead to gene expression, the repair proteins produced at the cell body can be supplied to spinal nerve injury site as well as peripheral target. According to this paradigm, intrinsic factors induced at the neuron itself may be primarily critical for axonal regeneration in both peripheral and central nervous systems.

Previous studies have shown that glial cells play a critical role in regulating axonal regeneration after nerve injury[16,17]. Schwann cell activation after peripheral nerve injury favors for axonal regeneration, whereas activation of oligodendrocytes or astrocytes inhibits axonal regeneration in the central nervous system[18]. After spinal cord injury, activated astrocytes, called reactive astrocytes, together with activated microglial cells secrete glial scar proteins such as chondroitin sulfate proteoglycan (CSPG) and inhibit axonal regrowth[16]. However, astrocyte-mediated glial scar formation can help axonal regrowth after spinal cord injury[19]. Recent studies suggested that vimentin-positive non-neuronal cell types which are induced at the injury site after spinal cord injury may provide permissive environment for axonal regeneration[20]. The latter study implies possible permissive function of non-neuronal cells for axonal regeneration. In our recent study, production of cell division cycle 2 (Cdc2) protein was induced in the spinal cord tissue after contusion injury, and Cdc2 further phosphorylated vimentin protein in cultured astrocytes (unpublished observation). Cdc2 is a prototypical cell cycle protein that mediates G2 to M phase transition in dividing cells[21]. In the injured nerves, Cdc2 activity appears to be associated with enhanced axonal regeneration after sciatic nerve injury and spinal cord injury[11,22]. Thus, it is conceivable that functional regulation of astrocytes could be one approach to induce regenerative responsiveness after spinal cord injury.

In the present study, we examined potential role of SJDBT on axonal regeneration using DRG single culture and co-culture systems. In DRG single culture system, effects of SJDBT treatment on neurite outgrowth in preconditioned DRG neurons were investigated. Preconditioned DRG neurons mimic in vivo sciatic nerve after injury, and as shown in Fig. 1, neurite elongation of preconditioned neurons exceeds much longer than non-preconditioned control cells, implicating regenerative responses of DRG neuron axons after sciatic nerve injury. Treatment of SJDBT into preconditioned cells further increased neurite outgrowth, suggesting that SJDBT may act on DRG neurons to induce enhanced axonal elongation. Alternatively, SJDBT might act on Schwann cells in the culture together with DRG sensory neurons. In any case, growth-promoting activity of SJDBT on DRG neurites could implicate similar physiological consequences occurring in the injured sciatic nerve in vivo.

We further investigated the effects of SJDBT on astrocytes in relation to neurite outgrowth in preconditioned DRG neurons. Primary non-neuronal cell culture was prepared from the spinal cord tissue 3 days after contusion injury. Among several types of non-neuronal cells, astrocytes are predominant in the spinal cord, and relative ratio of astrocytes to other cells is further increased after spinal cord injury. In our culture condition, astrocytes, identified by GFAP marker, were most abundant among all non-neuronal cell types, accounting 98% of Hoechst 33258-stained nuclei. We further found that 94% of GFAP-positive cells were positive to vimentin protein. It is thus possible that regenerative activity of non-neuronal cells can be investigated through the identification of vimentin signals in cultured cells. Our data showed that the addition of non-neuronal cells to DRG neurons inhibited neurite outgrowth when observed 24 hr later, displaying significant decreases in neurite length compared to single culture. However, neurite length after 48 hr of co-culture was increased to a similar level to that of DRG neuron single culture. These data suggest that non-neuronal cells after 48 hr culture might be permissive to neurite outgrowth of DRG neurons.

To investigate whether SJDBT directly acts on non-neuronal cells in culture, changes in vimentin protein

signals were determined by immunofluorescence staining in SJDBT-, SJH- or saline treated cells. Staining intensity of vimentin protein was much stronger in SJDBT-treated group than vehicle control or SJH-treated cells. Busch *et al.*[20] reported that vimentin-positive cells are localized at the epicenter after spinal cord injury and positively associated with regenerative activity of injured axons, and further showed that vimentin-positive cells at the injury area revealed increases in both protease activity and laminin and fibronectin production. This study indicates that increased vimentin production in non-neuronal cells may be functionally associated with axonal regeneration. In our experimental system, GFAP-positive astrocytes are almost exclusively vimentin-positive, suggesting that the astrocytes which were activated in co-culture system may provide further permissive environment for axonal regeneration mediated by SJDBT. It should also be noted that vimentin is one of Cdc2 kinase substrates, and increased Cdc2 phosphorylation of vimentin in cultured astrocytes was positively associated with increased regenerative responses of injured axons in the spinal cord. Taken together, it is feasible that SJDBT-induced increases of vimentin levels in astrocytes could be related to increased phosphorylation with Cdc2 kinase. Further investigation on the role of SJDBT in Cdc2 phosphorylation of vimentin may be helpful to understand the mechanism of neurite growth enhancement.

In summary, the present study shows that SJDBT appears to increase neurite outgrowth of preconditioned DRG neurons by acting directly on neurons or on glial cells in the injured nerves such as astrocytes and Schwann cells. Further studies on molecular mechanisms underlying the interaction with astrocytes may be useful to develop therapeutic strategies on axonal regeneration after spinal cord injury.

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