Growth Promoting Effects of Oriental Medicinal Drugs on Sciatic Nerve Regeneration in the Rat

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Oriental medicinal drugs have a broad spectrum of clinical use for the cure of nervous system diseases including brain ischemic damages or neuropathies. Yet, specific drugs or drug components used in the oriental medicine in relation to nerve fiber regeneration are not known. In the present study, possible growth promoting effects of oriental medicinal drugs were investigated in the injured sciatic nerve system in the rat. By immunofluorescence staining, we found that Jahageo (JHG, Hominis placenta) increased induction levels of axonal growth associated protein GAP-43 in the rat sciatic nerve. Small growth promoting activity was found in Golsebo (GSB, Drynariae rhizoma) and Baikhasuo (BHSO, Polygoni multiflori radix) drugs. JHG also increased cell cycle protein Cdc2 levels in the injured area of the sciatic nerves. Immunofluorescence staining indicated that induced Cdc2 protein was mostly localized in the Schwann cells in the injury area, implying that JHG activity might be related to increased Schwann cell proliferation during axonal regeneration. Moreover, levels of phospho-extracellular signal-regulated (ERK) pathway in the injured nerves were elevated by JHG treatment while levels of total ERK were unaltered. In vivo measurement of axonal regeneration using retrograde tracer showed that JHG, GSB and BHSO significantly enhanced DiI-labeled regenerating motor neurons compared with saline control. The present data suggest that oriental medicinal drugs such as JHG, GSB, and BHSO may be a useful target for developing specific drugs of axonal regeneration.

Key words : Hominis placenta, Polygoni Multiflori Radix, Drynariae Rhizoma, sciatic nerve, regeneration, GAP-43, Cdc2, ERK

Introduction

Peripheral axons in vertebrate animals can regenerate after nerve injury and accomplish its functional recovery[1,2]. Yet, the regeneration efficiency is not far from perfect because wrong targeting and retardation in axonal growth activity can occur and thus reduce the regeneration efficiency[3]. Numerous studies have revealed that diverse molecular factors are induced during axonal regeneration and their potential roles in axonal regeneration have been studied. Induction and potential role of adhesion molecules including immunoglobulin superfamily proteins (e.g., N-CAM, L1, and P0), neurotrophic factors (e.g., nerve growth factor, brain derived growth factor, ciliary neurotrophic factor, fibroblast growth factor), and axonal growth cone proteins such as synaptic vesicle-associated protein (e.g., synaptophysin, synaptagmin, synapsin) and axonal growth associated protein GAP-43[4-8] have been well demonstrated.

However, most of the studies on axonal regeneration have been primarily focused on elucidating endogenous molecular factors. Oriental medicinal drugs have a broad spectrum of clinical use including for the cure of cardiovascular and nervous system diseases, some of which have been implicated for functional recovery of nervous system after brain ischemic damages or neuropathies[9]. Despite the open possibilities of environmental molecular factors which could be beneficial for axonal regeneration, little is known about their effects on axonal regeneration. Among a few reports, Cheng et al.[10] and Xu et al.[11] have shown that herbal drug Buyang huanwu decoction had a growth promoting effect on the regenerating sciatic nerve, implicating potential role of oriental medicinal drugs in axonal regeneration. Ginsenoside Rb1 was also shown to be effective for peripheral nerve regeneration and also for the survival of injured spinal cord neurons[12,13]. Yet, specific drugs or drug components used in the oriental medicine in relation to nerve fiber regeneration

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are not known. In the present study, eight oriental medicinal drugs which are known to be effective for promoting renal function have been selected to examine potential role for peripheral axonal regeneration. According to oriental medical theory[14], 'The kidney produces the marrow'. The marrow can be classified into bone marrow, brain and spinal cord depending on what they supply, and sufficient essential energy makes the renal 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, ones that particularly strengthen muscles and bones were selected for the investigation. Through the primary screening of eight different drugs in sciatic nerve regeneration process, potentially useful drugs were examined by histochemical and biochemical analyses. Our data provide a compelling evidence on the role of promising oriental medicinal drugs on peripheral axonal regeneration.

Materials and Methods

1. Experimental animals

Rats (Sprague-Dawley, male 6-8 weeks old) 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. Only healthy animals were selected for experiments.

2. Preparation of oriental medicinal drugs

Following oriental medicinal drugs were used in the present study: Hominis Placenta (JHG; 紫河車), Cervi Pantotrichum Cornu (NY; 鹿茸), Cynomorii Herba (SY; 鎖陽), Polygoni Multiflori Radix (BHSO; 白何首烏), Cicadae Periostracum (ST; 蟬退), Drynariae Rhizoma (GSB; 骨碎補), Aconiti lateralis Preparata Radix (BJ; 附子), Taxilli Ramulus (SKS; 桑寄生). Drugs were 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°C, and dissolved in PBS. The stock solution was kept at -20℃ and used for experiment by diluting with physiological saline solution.

3. Sciatic nerve surgery

Animals were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg). Sciatic nerve was exposed and crushed with a pair of forceps held tightly for 30 sec twice at 1 min intervals[15]. Then, drugs in saline solution or the equal volume of saline were microinjected into the crush sites. Animals were recovered from anesthesia and sacrificed 3-14 days later. Animals were deeply anesthetized with a mixture of ketamine and xylazine, and sciatic nerves were separately dissected, immediately frozen, and kept at -70°C until use. For purpose of immunohistochemistry experiments, the sciatic nerves were prepared by dividing into the proximal stump, 5-mm segments proximal to the injury site, and the distal stump, 5-mm segments distal to the injury site.

4. Histology and immunofluorescence staining

Nerve segments were embedded and frozen at -20°C. 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) 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. 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 sections from the individual experiments, 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 μg/ml leupeptin, 5 μg/ml 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 unspecific 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℃. The membrane was washed and incubated for one and half hour at room temperature in a solution containing a 1:1000 dilution of polyclonal antibodies specific for the C-terminal of rat GAP-43, monoclonal antibodies specific for C-terminal of rat Cdc2 or the antibodies to detect ERK protein or phosphorylated ERK protein. 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. Retrograde tracing of motor neurons in the spinal cord

The sciatic nerve of anesthetized rats with ketamine and xylazine was exposed and DiI (5 $\mu\ell$ of 3 % in dimethylsulfoxide Molecular Probes) was applied to 10 mm distal to the injury site with a microsyringe. The incision was sutured, and the animals were returned to their cages after recovered from the narcosis. Forty eight hours later, animals were sacrificed and DiI-labeled motor neurons in the longitudinal spinal cord sections were visualized and counted under fluorescence microscope by an observer unaware of the experimental treatments. The mean numbers of total labeled cells in individual animals were compared among groups by paired Student's t-test.

Results

1. Screening of the oriental medicinal drugs

To determine the pattern of axonal regeneration, prepared sections were double immunostained with neurofilament-200 (NF-200) and GAP-43 antibodies. NF-200 is selectively expressed in the neuronal cells, particularly in the axon and thus used as axon specific marker. GAP-43 is highly induced in regenerating axons. As shown in the representative data on the pattern of double immunofluorescence staining in Figure 1, Jahageo (JHG), Golsebo (GSB), and Baikhasuo (BHSO) showed strong immunostaining signals for GAP-43 (in red), and the other 5 drugs examined did not show any changes in GAP-43 immunoreactivity (data not shown). Immunostaining intensity of GAP-43 was highly induced in both proximal and distal portions from the crush site of the nerve. Induction levels of GAP-43 protein in nerve treated with BHSO or GSB were lower compared with nerves treated with JHG, but the comparison with saline injected control side was clearly higher.

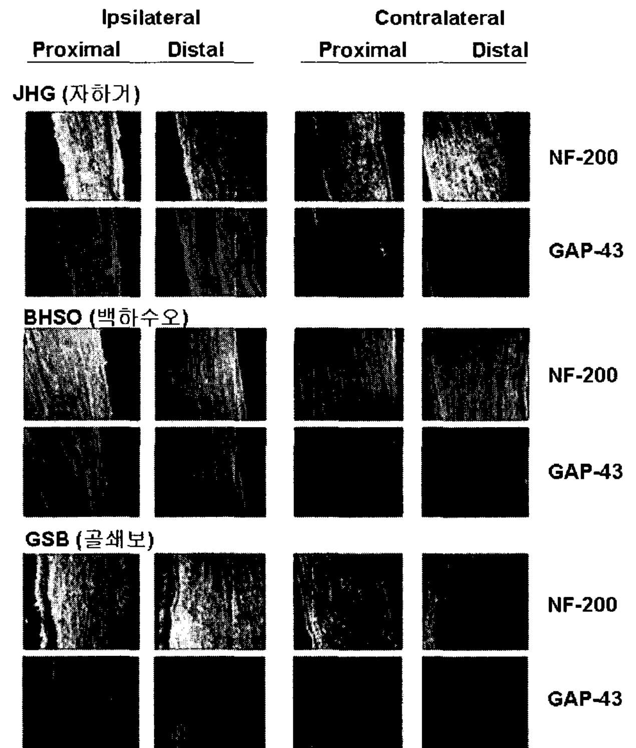

Fig. 1. Immunofluorescence staining of injured sciatic nerves. Sciatic nerves from individual rats were crushed and 5 μl drugs (10 mg/ml) were injected into the injury site. 7 days later, proximal and distal nerve sections were prepared for double immunoflurescence staining with anti NF-200 and GAP-43 antibodies. Signals for NF-200 and GAP-43 were visualized with fluorescein (green) and rhodamine (red) respectively. Ipsilateral indicates crushed nerves with drug treatment and contralateral indicates saline injected crushed nerves.

2. Effects of oriental medicinal drugs on GAP-43 expression in the injured nerves

To determine GAP-43 expression, GAP-43 protein levels were determined by Western blotting in crushed nerves after drug treatment. As shown in Fig. 2, saline treated nerves showed GAP-43 protein induction only in the proximal nerve segment. GAP-43 protein was much increased in crushed nerves with JHG treatment compared with saline injected crushed nerves. Comparison between proximal and distal nerve segments was similar though slightly increases in the distal nerve were observed. Nerves treated with GSB showed relatively increased GAP-43 protein in the distal nerves. In

nerves treated with BHSO, GAP-43 protein was relatively high in proximal compared to distal nerves. In nerve sample with no crush or with JHG, GSB or BHSO treatment alone, no GAP-43 protein expression was detected (data not shown).

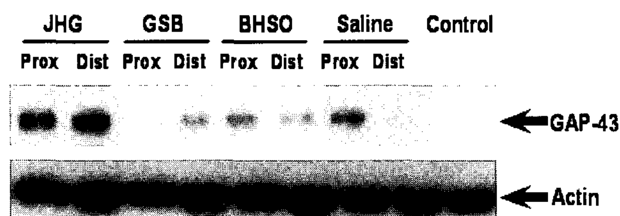

Fig. 2. Western blot analysis of GAP-43 protein levels in the sciatic nerve after various treatments. At the time of sciatic nerve crush, JHG, GSB, or BHSO was injected into the injury site. Proximal (Prox) or distal (Dist) nerve segments from the injury site were separately prepared and used for cell lysate preparation and GAP-43 Western blotting. Western blotting with anti-actin antibody was done as an internal loading control.

3. Effects of oriental medicinal drugs on Cdc2 protein levels in regenerating sciatic nerves after injury

Cell division cycle 2 (Cdc2) kinase is known to play a critical role in cell cycle progression, but its function in post-mitotic neuron is largely unknown. To examine whether drug treatment affects on Cdc2 protein expression in crushed nerves, sciatic nerves were crushed with or without drug injection, and nerves were prepared 7 days later for Western analysis or for immunofluorescence staining. As shown in Fig. 3, JHG-treated crushed nerves showed slightly higher levels of Cdc2 protein expression than saline-treated nerves. Similar levels of Cdc2 protein between GSB or BHSO-treated nerves and saline treated groups were observed. In nerve with no surgery, Cdc2 protein was not detected.

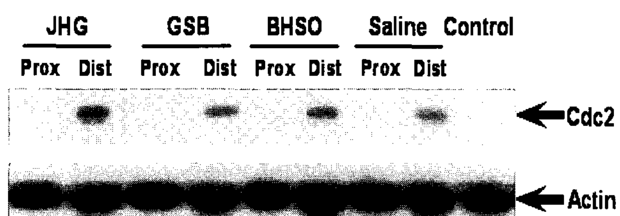

Fig. 3. Western blot analysis of Cdc2 protein levels in the sciatic nerve after various treatments. At the time of sciatic nerve crush, JHG, GSB, or BHSO was injected into the injury site. Proximal (Prox) or distal (Dist) nerve segments from the injury site were separately prepared and used for cell lysate preparation and Cdc2 Western blotting. Western blotting with anti-actin antibody was done as an internal loading control.

To determine whether Cdc2 protein is induced in neuronal or non-neuronal cells such as Schwann cells, Cdc2 immunostaining was performed with neuron-selective marker protein NF200 or with Schwann cell marker protein S100β protein. It is observed that most of the Cdc2 signals were not overlapped with NF-200 signals, but with S100β protein signals (Fig. 4A), strongly suggesting that induced Cdc2 protein was expressed in the Schwann cells.

To determine whether increased Cdc2 protein is related to Schwann cell function, sciatic nerve sections were stained with Hoechst dye. Hoechst dye 33258 specifically interacts with DNA, and thus is used for nuclear staining. Comparison of nuclear staining among non-injured sciatic nerves and injured nerves in the presence or the absence of JHG treatment revealed that injury induced significant elevation in a number of stained nuclei in the injury site of the sciatic nerves (Fig. 4B). The number of nuclei was further increased by JHG treatment, Together, these data suggest that induced Cdc2 protein in the injured sciatic nerves was further up-regulated in proliferating Schwann cells.

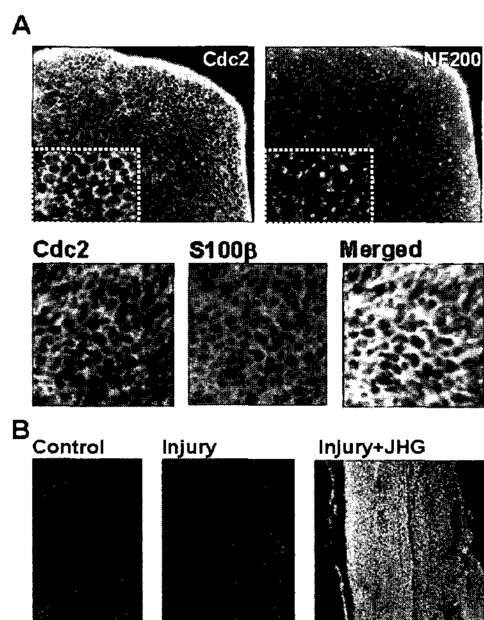

Fig. 4. In vivo distribution of Cdc2 protein in the sciatic nerves. (A) The sciatic nerves prepared 7 days after injury were used for the cross section preparation. (Upper panel). One set of sections prepared from the sample treated with both nerve injury and JHG treatment was used for immunofluorescence staining with anti-Cdc2 antibody and another set for neurofilament-200 (NF-200) staining. (Lower panel). Sciatic nerve sections after the injury and JHG treatment were used for double-immunofluorescence staining with Cdc2 and S100β antibodies. Photographic images at higher magnification show that Cdc2 signals were distributed peripherally to the area where neurofilament protein signals were densely stained, and mostly merged onto the S100β signals. (B). Hoechst staining of sciatic nerves. Sciatic nerves were prepared from non-treated control, or 7 days after crush or after crush and JHG treatment. Longitudinal nerve sections were used for Hoechst 33258 dye staining for 10 min, and nuclei shown in blue under fluorescence microscope were compared among three groups.

4. Effects of oriental medicinal drugs on ERK1/2 MAP kinase activation in regenerating sciatic nerves after injury

To examine the involvement of ERK MAP kinase in axonal regeneration and also if their activities are further changed in response to drugs, protein lysates of sciatic nerves after surgery and/or drug treatment were analyzed by Western blotting. Although total ERK protein levels in sciatic nerves were not changed among groups with various treatments, it was found that phospho-ERK protein was strongly induced in sciatic nerves 7 days after injury (Fig. 5,

upper panel). In injured nerves, treatments of JHG elevated phospho-ERK protein levels in both proximal and distal nerve segments from the crush site. Treatments with GSB or with BHSO showed similar levels of phospho-ERK protein compared with saline treated nerves though there were some variations in protein levels between proximal and distal portions of nerves. These data indicate that activation of ERK activity in the injured nerves was augmented by JHG treatment while levels of total ERK were unaltered.

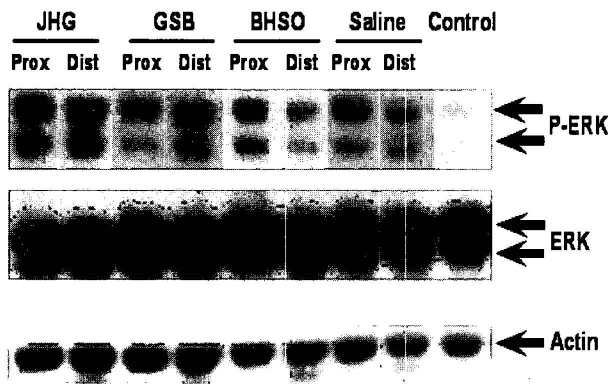

Fig. 5. Levels of total ERK1/2 and active ERK in the sciatic nerves. During sciatic nerve injury, drugs were injected and cell lysates were prepared 7 days later. Proximal (Prox) and distal (Dist) segments to the injury site were separately prepared and used for Western analysis with anti-phospho-ERK1/2 antibody or with anti-ERK1/2 antibody to determine levels of active or total ERK1/2 protein levels. Western analysis with actin antibody was performed as an internal loading control.

5. Effects of oriental medicinal drugs on sciatic nerve regeneration after injury; retrograde tracing analysis

Sciatic nerve was crushed, and 5 days later, DiI was injected into the nerve 10 mm distal to the original injury site to allow diffusion into the motor neuronal cell bodies which are located in the spinal cord. In an animal group with no nerve injury, DiI injection into the nerve can label motor neuron cell bodies as labeled 'no injury control' in Fig. 6A. In a group with sciatic nerve injury, the number of DiI-labeled motor neurons was much lower compared to no injury control group. In an animal group treated with JHG when nerve injury was provided, the number of DiI-labeled neurons was significantly elevated reaching to the similar levels of no injury control. The number of labeled neurons in animal groups treated with GSB or BHSO was slightly increased above injury group, but lower than JHG treated group.

To confirm microscopic observation, all DiI-labeled cells from individual sections were summated, and statistical comparison was made. As shown in Fig. 6B, number of diI labeled cells in JHG, BHSO, and GSB treated injured neurons was significantly increased compared to that of injury only group. These data suggest that JHG was effective in promoting sciatic nerve fiber regeneration after crush injury.

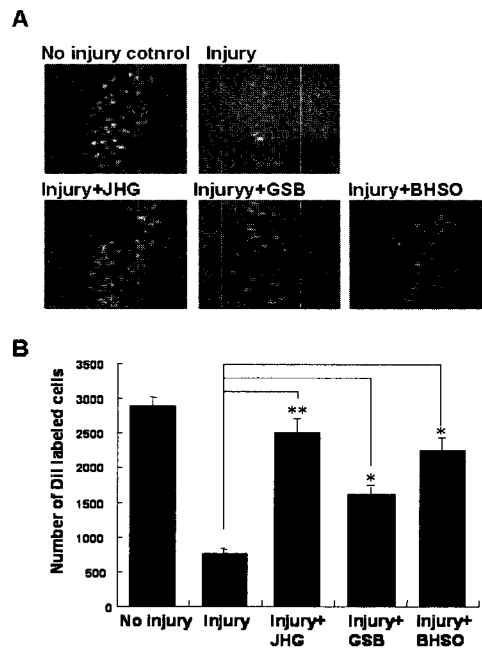

Fig. 6. Retrograde labeling of motor neuron cell bodies in the spinal cord. (A) Individual animal's sciatic nerves were subject to various treatments as described in the Figure, and cross sections of the spinal cord at the lower thoracic level were prepared. DiI-stained sections (in red) were observed under fluorescence microscope. (B) Comparison of dil-labeled cells in the spinal cord of rats with various treatments. The bar graph represents mean ± SEM (n = 4). * $p < 0.05$, ** $p < 0.01$ (paired Student's t-test).

Discussion

The major objective for the present study was to examine growth promoting effects of oriental medicinal drugs on regenerating axons. By treating 8 different drugs on the injury site of the sciatic nerves, we found that JHG, GSB and BHSO were most effective in inducing axonal regeneration marker protein GAP-43. The selected drugs, particularly JHG had the highest growth promoting effects on injured sciatic nerve when examined by retrograde tracing analysis. Moreover, JHG treatment induced axonal regeneration related proteins such as Cdc2 protein and active form of ERK protein. Thus, these selective oriental medicinal drugs may be important for axonal regeneration process after injury.

Several lines of our data strongly suggest a potential function of JHG as a regeneration-inducing exogenous factor. First, axonal growth protein GAP-43 was highly induced in the sciatic nerves after injury. GAP-43 has been studied extensively since it was identified in regenerating axons of the rabbit and the toad[7,8,16-18]. Second interesting finding from the present study is the up-regulation of Cdc2 protein in the injured nerves with JHG treatment. Cdc2, also called cyclin-dependent kinase 1 (Cdk1), is dependent on Cyclin B for its activity as its name implies. Cdc2 is known to be involved in the progression of G2 to M phase in the cell cycle[19]. Several Cdk family proteins including Cdc2 were shown to be involved in

apoptosis of neuronal cells[20]. It was reported that Cdc2 activation was specifically involved in cerebellar neuron apoptosis via the activation of proapoptotic protein Bad[21,22]. In the present study, regenerating nerves showed clear and strong induction of Cdc2 protein in the sciatic neve system as demonstrated in the facial nerves[23]. It is not clear yet whether Cdc2 protein is induced in the regenerating axons or neighboring non-neuronal cells such as Schwann cells or both. Considering that the levels of Cdc2 protein induced in the injured nerve region are high, involvement of Schwann cells is highly probabilistic. In relation to this argument, it should be noted that bulged injury area are formed in the injury site 3-7 days after surgery and returned to normal shape 14 days later, and changes in Cdc2 protein levels follow the similar time window. Also, Hoechst staining showed increased density of nuclei and increased S100β protein staining in the injury area. Since Schwann cells are important for guiding growing axons toward its target muscle[24], it can be speculated that one possible role of induced Cdc2 would be to help Schwann cells promote axonal regeneration. JHG increased Cdc2 protein levels in the injured nerve, suggesting that these drugs might act as a positive regulator for growth promoting effects of Schwann cells. Further studies, for example by using Cdc2 inhibitor to inhibit Schwann cell proliferation, would be required to reveal its precise role in axonal regeneration process.

Third finding is that the treatment of JHG drug slightly increased the phospho-ERK protein. It was also noted that GSB and BHSO treatment did not change phospho-ERK protein, suggesting that JHG might be closely associated with axonal regeneration than other drugs examined. Mitogen activated protein kinase (MAP kinase) is a family of protein that is regulated by a series of upstream kinases which begins its original external signaling vis small GTPase protein in the cell membrane[25]. MAP kinases are classified into ERK, JNK and p38. ERK MAP kinase is known to be important for cell survival whereas p38 and JNK kinases play an important role in apoptosis[26]. ERK is primarily important for transmitting signal transduction to induce cellular response, and thus its induction could be transient. ERK has several isoforms, and yet, only ERK1 and 2 have been largely studied. A few studies have reported activation of ERK in regenerating axons[27]. It was recently reported that ERK activation can induce Cdk5 kinase activation via up-regulation of p35 regulatory protein leading to FAS activation and promote axonal growth[28]. All together, the present study strongly implicate that Hominis Placenta (JHG; 紫河車) may be directly or indirectly involved in growth promoting effects on regenerating axons. Increased labeling of sciatic nerve motor neurons by JHG, GSB, and BHSO after sciatic nerve injury supports this interpretation. As we consider the effect of herbal medicine, Polygoni Multiflori Radix (BHSO; 白何首烏) is stronger in replenishing Yin and blood than others, Drynariae Rhizoma (GSB; 骨碎補) is more effective in promoting blood circulation, and JHG plays a critical role in promoting essential energy. Therefore, the fact that drugs promoting Yin and blood are more effective in axonal regeneration can be inferred.

JHG (Hominis placenta; 紫河車), as its name implies, is a drug of human placenta[29]. In fact, it has been used as a medicinal stuff for a long time in oriental medicine. Many researchers have reported on its effects in many areas such as pulmonary tuberculosis, asthma, sterility, psychiatric disorders and so on[29]. However, no studies have been reported about JHG in relation to axonal regeneration. According to herbology literature, the major components of JHG are estradiol, progesterone, acetylglucosamine, D-galactose, mannose, and amino acids[29]. Yet, since placenta was used for drug preparation, all biological molecules should be contained except the most of the active forms of protein loss by denaturalization or degradation. The degradation of most of the proteins in the JHG samples used in this study was confirmed by SDS-PAGE (data not shown). It would be a formidable task to identify the single compound effective for axonal regeneration.

One point that is worth mentioning is that since new born animals including human are much more active in neuronal activity in terms of proliferation and differentiation, a certain molecular factors which could exist in the placenta may play a role in axonal growth promoting activities. Further studies on axonal regeneration by different drugs that replenish Yin and blood should be done, and the different effect between localized injection and oral medication on axonal regeneration needs to be proved by further investigation.

References

1. Fawcett, J.W., Keynes, R.J. Peripheral nerve regeneration. Annu Rev Neurosci 13, 43-60, 1990.
2. Ide, C. Peripheral nerve regeneration. Neurosci Res 25, 101-121, 1996.
3. Al-Majed, A.A., Neumann, C.M., Brushart, T.M., Gordon, T. Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration. J Neurosci 20, 2602-2608, 2000.
4. Lindsay, R.M., Wiegand, S.J., Altar, C.A., DiStefano, P.S. Neurotrophic factors: from molecule to man. Trends Neurosci 17, 182-190, 1994.

5. Curtis, R., Adryan, K.M., Zhu, Y., Harkness, P.J., Lindsay, R.M., DiStefano, P.S. Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury. Nature 365, 253-255, 1993.
6. Fu, S.Y., Gordon, T. The cellular and molecular basis of peripheral nerve regeneration. Mol Neurobiol 14, 67-116, 1997.
7. Skene, J.H.P., Willard, M. Characteristics of growth associated polypeptides in regenerating toad retinal ganglion 'cell axons. J Neurosci 1, 419-426, 1981.
8. Skene, J.H.P., Willard, M. Axonally transported proteins associated with axon growth in rabbit central and peripheral nervous system. J Cell Biol 89, 96-103, 1981.
9. Kim Y.S., Kim D.H., Seol I.C., Kim B.T. Study on the effect of Chukdamtang and Kamichukdamtang extract on brain damage and thromboembolism. Korean J Oriental Physiol & Pathol 15(1):92-104, 2001.
10. Cheng, Y.S., Cheng,W.C., Yao, C.H., Hsieh, C.L., Lin, J.G., La,i T.Y., Lin, C.C. Effects of buyang huanwu decoction on peripheral nerve regeneration using silicone rubber chambers. Am J Chin Med 29, 423-432, 2001.
11. Xu, H., Jiang, B., Zhang, D., Fu, Z., Zhang, H. Compound injection of radix Hedysari to promote peripheral nerve regeneration in rats. Chin J Traumatol 5, 107-111, 2002.
12. Chen, Y.S., Wu, C.H., Yao, C.H., Chen, C.T. Ginsenoside Rb1 enhances peripheral nerve regeneration across wide gaps in silicone rubber chambers. Int J Artif Organs 25, 1103-1108, 2002.
13. Liao, B., Newmark, H., Zhou, R. Neuroprotective effects of ginseng total saponin and ginsenosides Rb1 and Rg1 on spinal cord neurons in vitro. Exp Neurol 173, 224-234, 2002.
14. Kim W.H., Choi W.Y. 臟腑辨證論治. 서울, 成輔社, p 281-287, 1990.
15. Namgung, U., Routtenberg, A. Transcriptional and post-transcriptional regulation of a brain growth protein: regional differentiation and regeneration induction of GAP-43. Eur J Neurosci 12, 3124-3136, 2000.
16. Bomze, H.M., Bulsara, K.R., Iskandar, B.J., Caroni, P., Skene, J.H. Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons. Nat Neurosci 4, 38-43, 2001.
17. Benowitz, L.I., Routenberg, A. A membrane phosphorylation associated with neuronal development, axonal regulation, phospholipid metabolism and synaptic plasticity. Trends Neurosci 10, 527-531, 1987.
18. Gispen. W.H., Boonstra, J., De Graan, P.N.E., Jennekens, F. G.I.. Oestreicher, A.B., Schotman, P., Schrama, L.H., Verhaagen, J. and M argolis, F.L. B-50/GAP-43 in neuronal development and repair. Restorat Neurol Neurosci 1, 237-244, 1990.
19. Dorée, M., Galas, S. The cyclin-dependent protein kinases and the control of cell division. FASEB J 8, 1114-1121, 1994.
20. O'Hare, M., Wang, F., Park, D.S. Cyclin-dependent kinases as potential targets to improve stroke outcome. Pharmacol Ther 93, 135-143, 2002.
21. Konishi, Y., Lehtinen, M., Donovan, N., Bonni, A. Cdc2 phosphorylation of BAD links the cell cycle to the cell death machinery. Mol Cell 9, 1005-1016, 2002.
22. Konishi, Y., Bonni, A. The E2F-Cdc2 cell-cycle pathway specifically mediates activity deprivation-induced apoptosis of postmitotic neurons. J Neurosci 23, 1649-1658, 2003.
23. Namgung, U., Choi, B.H., Park, S., Lee, J.U., Seo, H.S., Suh, B.C., Kim, K.T. Activation of Cyclin-dependent kinase 5 is involved in axonal regeneration. Mol Cell Neurosci 25, 422-432, 2004.
24. Son, Y.J., Thompson, W.J. Schwann cell processes guide regeneration of peripheral axons. Neuron. 14, 125-132, 1995.
25. Davis, R.J. Signal transduction by the JNK group of MAP kinases. Cell 103, 239-252, 2000.
26. Namikawa, K., Honma, M., Abe, K., Takeda, M., Mansur, K., Obata, T., Miwa, A., Okado, H., Kiyama, H. Akt/protein kinase B prevents injury-induced motoneuron death and accelerates axonal regeneration. J Neurosci 20, 2875-2886, 2000.
27. Kishino, A., Nakayama, C. Enhancement of BDNF and activated-ERK immunoreactivity in spinal motor neurons after peripheral administration of BDNF. Brain Res 964, 56-66, 2003.
28. Desbarats, J., Birge, R.B., Mimouni-Rongy, M., Weinstein, D.E., Palerme, J.S., Newell, M.K. Fas engagement induces neurite growth through ERK activation and p35 upregulation. Nat Cell Biol 5, 118-125. 2003
29. 전국한의과대학 본초학교수 共編著. 本草學. 서울, 永林社, pp 155-156, 287-288, 331-333, 545-546, 562-563, 567-568, 571-572, 583-584, 2000.