Introduction

Peripheral nerve axons have the ability to regenerate and reinnervate the original target leading to functional recovery. This property is attributed to both intrinsic neuronal property and environmental influences\(^1\sim3\). Many molecular factors known to be induced in the injured nerve can be regulated locally at the injury site or at the nucleus via the induction of target gene expression. For instance, axonal growth-associated protein GAP-43, which is increased in the injured axons, is induced at gene expression levels in response to lesion signal transmitted retrogradely from the axonal injury site\(^4,5\). Schwann cells are known to play an active role for axonal regrowth. They, together with macrophages, are involved in phagocytotic activity to remove Wallerian degenerated tissue debris and provide a guide for axonal elongation to the distal area\(^3\). Schwann cells also produce diverse neurotrophic factors whose interaction with injured axons generates lesion signal for regeneration.

It seems thus possible that the supply of both intrinsic and environmental molecular factors into the injured nerve promotes peripheral axonal regeneration. Indeed, overexpression of GAP-43 protein and neurotrophic factors was shown to facilitate spontaneous axonal sprouting to innervate to skeletal muscle\(^6\). Yet, there have been reports implicating that peripheral nerve regeneration would require additional inputs in order to achieve correct targeting and functional recovery\(^7\). Successful regrowth, but incorrect targeting of motor and sensory axons, in the peripheral nerve can result in a functional deficit\(^8\). It was reported that the electric stimulation of injured nerve improved correct targeting of regenerating axons, and physical exercise in experimental rats enhanced axonal regeneration accompanying functional recovery\(^3,8\).

While an effort to identify the regeneration factors and increase axonal regeneration has so far been mostly concentrated on endogenous agents, there are increasing evidences that exogenous agents such as herbal products can enhance axonal regeneration. Here in this present study, Sengmaek-san (Shengmai-san; SMS) was investigated whether it could improve axonal regrowth in the injured sciatic nerve. SMS, together with several other herbal medicine, has been used for treating cardiovascular and neurological disorders and other abnormal body symptoms\(^11\). Recent studies showed that SMS can play a protective role in cells damaged by free radicals or reactive oxygen species\(^12,15\), and also protect PC12

Key words : Sengmaek-san(Shengmai-san), sciatic nerve, axonal regeneration, GAP-43, phospho-Erk1/2

Sengmaek-san(Shengmai-san; SMS) is used in oriental medicine as one of the key herbal medicine for treating diverse symptoms including cardiovascular and neurological disorders. In the present study, the effects of SMS on axonal regeneration were investigated in the rat model given sciatic nerve injury. SMS treatment enhanced axonal regrowth into and the number of non-neuronal cells in the distal area after crush injury. GAP-43 protein levels were increased in the injured sciatic nerve compared to intact nerve and further upregulated by SMS treatment. GAP-43 protein was increased similarly in the dorsal root ganglion (DRG) at lumbar 4 - 6 by nerve injury and SMS treatment, suggesting GAP-43 induction at gene expression level. SMS-mediated increase in phospho-Erk1/2 protein was observed in the DRG as well as in the injured nerve implying its retrograde transport into the cell body as the process of lesion signal transmission. The present findings suggest that SMS may be involved in enhanced axonal regeneration via dynamic regulation of regeneration-associated proteins.
cells from amyloid-β-induced cytotoxicity possibly by anti-oxidative mechanism\textsuperscript{16}. Since neuroprotection via anti-oxidative reactions might affect axonal elongation in some pathophysiological situations such as amyotrophic lateral sclerosis (ALS) and other neuropathies (e.g., diabetes and dry beriberi\textsuperscript{17,18}), SMS could mediate growth-promoting process in regenerating peripheral axons. To examine this possibility, effects of SMS on axonal regeneration were evaluated using histological and biochemical methods in the rats given sciatic nerve injury. Our study demonstrates that SMS acts positively for enhanced axonal regeneration accompanying the induction of regeneration-associated proteins such as GAP-43 and Erk1/2 proteins.

Materials and Methods

1. Materials

1) Experimental animals

Sprague-Dawley rats (male, 8 weeks old, 200-250 g) were used in this experiment. They were placed in an animal room with regulated temperature (22°C), 60% of humidity, and 12-h light and 12-h dark cycle. They were allowed to eat commercial rat chow (Samyang Co., Korea) and drink water ad libitum.

2) Drugs

Dried SMS was obtained from Oriental Hospital of Daejeon University (Daejeon, Korea). Dried drugs (; Panax Ginseng 4 g, Ophiopogon Japonicus 8 g, Schisandra Chinensis 4 g)\textsuperscript{19} were resuspended in 2 liters of water, heat-extracted with 2 liters of water for 3 h, and filtered three times. Concentrated solutions were frozen at -70°C for 4 h, and freeze-dried for 24 h. The yield for SMS was 1.5 g for 16 g of the initial raw materials. Purified drug was kept at 4°C, and dissolved in water. The stock solution was stored at -20°C and used for experiment by diluting with physiological saline before use.

2. Experimental procedures

1) Sciatic nerve surgery procedure

Rats 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. Then, 5 μl SMS (10 mg/ml) in saline or the equal volume of saline was applied by dropping into the crush sites. Rats were recovered from anesthesia and sacrificed 7 days later. Rats 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.

2) Immunohistochemistry

For immunohistochemistry experiment, dissected tissues were frozen immediately at -75°C and embedded into the OCT medium. Dorsal root ganglion (DRG) was prepared at lumbar 4 - 6, and sciatic nerve were prepared at the injured area. The sections (20 μm) were cut using a cryostat and mounted on positively charged slides. Sections were fixed with 4% paraformaldehyde, 4% sucrose in phosphate-buffered saline (PBS) at room temperature for 40 min, permeabilized with 0.5% nonidet P-40 (NP-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 primary antibody, and 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. Primary antibody reaction was performed with single or double antibodies depending on the experimental purposes, and followed by 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. Tissue sections were treated with 25 μg/ml of Hoechst 33258 (Sigma) in 0.1% triton X-100 in phosphate-buffered saline solution (PBST) for 10 min. 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). The primary antibodies used for immunofluorescence staining were anti-NF-200 antibody, anti-GAP-43 antibody, and anti-tubulin antibody(Tuj1).

3) Western blot analysis

Nerve tissues were washed with ice-cold PBS, and sonicated under 50 - 200 μl 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 Na3VO4, 1% triton X-100, 10% glycerol, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 3 μM 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, Bedford, USA). Blots were blocked with 5% nonfat dry milk in PBST (17 mM KH2PO4, 50 mM Na2HPO4, 1.5 mM NaCl, pH 7.4, and 0.05% Tween-20) for 1 hr at room temperature and then incubated overnight at 4°C in 0.1% triton X-100 in PBS plus 5% nonfat dry milk containing antibodies. Protein bands were detected using the Amersham ECL kit (Amersham Pharmacia Biotech, USA), with horseradish peroxidase-conjugated secondary goat anti-rabbit or goat anti-mouse antibodies (Transduction Laboratories,
Results

Axon regeneration was analyzed by NF-200 immunofluorescence staining of longitudinal sciatic nerve sections. In the injured nerve treated with saline, NF-200-stained axons were clearly seen at the injury site, but the staining intensity was gradually decreased at the distal area from the injury site (Fig. 1). Axon staining at the distal nerve was much improved by SMS treatment although the staining intensity was lower than that in the intact nerve.

The number of non-neuronal cells along the sciatic nerve was investigated by Hoechst nuclear staining. The number of nuclei in defined areas along different distances from the injury site was much higher in saline-treated injured nerve than that in the intact sciatic nerve (Fig. 2A). Comparison between saline-treated and SMS-treated groups showed characteristic differences along the nerve. In saline-treated group, the number of cells decreased as moved toward the distal end. In contrast, overall cell number in SMS-treated group was higher than saline-treated group, and showed a tendency to increase at the distal nerve. Comparison at different position between saline and SMS-treated groups showed significant difference except at the injury site (Fig. 2B).

To determine the effects of SMS treatment on the synthesis of axonal growth-associated protein GAP-43 in the injured sciatic nerve, GAP-43 was analyzed by Western and immunofluorescence analyses. GAP-43 was not present in the intact sciatic nerve and then strongly induced after nerve injury (Fig 3A). SMS treatment further increased protein levels. Longitudinal distribution of GAP-43 in the sciatic nerve was investigated by immunofluorescence staining. In the

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Fig. 1. Enhanced axonal regeneration in SMS-treated sciatic nerves. Immunofluorescence staining of sciatic nerves with anti-NF-200 antibody. Seven days after crush injury, individual nerve segments at different distances from the injury site were used for the preparation of 20 μm nerve sections. Fluorescence images at different length from the injury site were observed and the represented images were shown in the figure.

Fig. 2. Increased cell numbers in regenerating sciatic nerves by SMS treatment. The nerve sections were prepared 7 days after crush injury for analysis. (A) The nuclei in the sections were stained with Hoechst 33258 dye and visualized in blue under the fluorescence microscope. (B) The number of Hoechst-stained nuclei were quantitatively analyzed in defined area as shown in (A). Mean number of Hoechst-stained nuclei for intact group was 384 ± 29. The error bars in the graph are mean ± SEM (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001 (Student t-test).

Fig. 3A: Longitudinal distribution of GAP-43 in the sciatic nerve.
saline-treated injured nerve, GAP-43 signals were particularly high in the area close to the injury site, but greatly reduced at the distal portion (Fig. 3B). In SMS-treated sciatic nerve, GAP-43 signals were evenly high along the nerve up to 10 mm portion distal to the injury site. The merged images showed that most of GAP-43 and NF-200 signals were overlapped and indicated by arrows in the figure (Fig. 3C). Yet, some GAP-43 signals were not overlapped with NF-200 signals as identified in red color, indicating that GAP-43 protein was produced from non-neuronal cells as well as neurons.

To determine whether induced GAP-43 is regulated at gene expression level, GAP-43 protein was investigated at DRG at lumbar 4-6 where the sensory neuronal cell bodies for sciatic nerve axons are located. As shown in Fig. 4A, GAP-43 protein was not observed in the DRG in the intact control animal, and then induced by sciatic nerve injury. Slightly further increase was observed by SMS treatment. Tissue distribution of induced GAP-43 protein in the DRG was examined by double immunostaining with anti-GAP-43 and anti-tubulin antibodies. In the intact animals, no GAP-43 protein signal was detected (Fig. 4B). In both saline- and SMS treated groups, GAP-43 protein signals were clearly observed in DRG neurons, and merged images showed high levels of overlapping between protein signals.
To determine whether axonal regeneration after sciatic nerve injury and SMS treatment involved regulation of active form of Erk1/2, protein, lysates from sciatic nerve and DRG were used for Western blot analysis by phospho-Erk1/2 protein. Phospho-Erk1/2 was found in the intact sciatic nerve, and increased by nerve injury(Fig. 5A). SMS treatment further elevated phospho-Erk1/2 levels in the injured nerve tissues. In the DRG, basal levels of phospho-Erk1/2 protein were detected and further increased after injury(Fig. 5B). Similar levels of phospho-Erk1/2 was observed between saline and SMS treated group.

Discussion

Sengmaek-san(SMS) was first recorded in ‘Dongwonshipsuh (東垣十書)’, and according to the oriental medicinal theories, it is effective for invigorating the vital energy and producing and converging the vital essence\(^{(1)}\). ‘Donguibogam (東醫寶鑑)’ describes that the vital energy is the original source for essence and spirit of the body that maintains its life activities. It also states that the vital energy becomes a commander of blood\(^{(28)}\). In other words, blood circulation depends on the activities of vital energy in driving and generating blood as well as keeping blood flowing within the vessels. The vital energy in motion renders blood circulate normally, and stagnation of the vital energy usually leads to blood stasis. When it is sufficient, the ability to make blood is strong because it is the motive power for generating blood, whereas when it is deficient, the blood is insufficient. Therefore, pathological condition of the vital energy causes numerous symptoms\(^{(2)}\).

SMS is believed to supply both the vital energy and essence\(^{(2)}\), and thus is used for the treatments of cardiac disorders, shock, or depletion of the vital energy. Recently studies using experimental animals revealed that SMS is effective for protecting cells from oxidative damage or lowering inflammatory cytokines and nitric oxide formation\(^{(16,29)}\). Neuronal damages caused by amyloid-β treatments were shown to be reduced by SMS treatment in cultured neuronal cells\(^{(16)}\).

Our histological examination revealed that SMS treatment on sciatic nerves after injury promoted axonal regrowth to the distal area. Longitudinal distribution of NF-200-labeled axons in the injured sciatic nerve showed that SMS treatment increased distal elongation in comparison to saline-treated group. Hoechst nuclear staining revealed significant increases in the cell number in the injured area after nerve injury. Since Schwann cell is the major cell type in the peripheral nerve, increased cell population might be mostly contributed by Schwann cell proliferation. Previous studies have shown that increased proliferation of Schwann cells in the injured nerve plays a supportive role for axonal regeneration\(^{(23)}\). Schwann cells after nerve injury function to remove degenerating cell debris, provide neurotrophic factors, and remyelinate newly growing axons. Thus, increased cell proliferation after injury and further upregulation by SMS treatment can be considered as one possible mechanism underlying SMS-mediated facilitation of peripheral axonal regrowth.

The present study also provides evidence that SMS-mediated axonal regeneration involves biochemical control of axonal growth-associated protein synthesis. GAP-43 is a widely accepted marker protein for axonal regeneration whose synthesis is increased in the injured nerve, particularly in the peripheral nervous system\(^{(5)}\). GAP-43 is primarily expressed in the neurons, and also is produced from Schwann cells\(^{(36)}\). Moreover, induction of GAP-43 expression in the brain is believed to be related to axonal sprouting after injury and synaptic plasticity\(^{(29)}\). The present data showed that GAP-43 was strongly induced in the injured sciatic nerve after injury. Although overall comparison of GAP-43 protein level within 1 cm nerve segment did not show any obvious difference between saline-treated and SMS-treated groups, segmental comparison from proximal to distal areas revealed stronger GAP-43 signals in SMS-treated group compared to saline control. Merged image for GAP-43 and NF-200 immunofluorescence staining showed largely overlapping signals of two proteins, suggesting that GAP-43 is expressed in neurons which have their soma in the DRG for sensory axons and in the ventral horn of the spinal cord for motor axons. Enhancement of axonal GAP-43 levels could be induced at gene expression level and/or by posttranslational activity, i.e., increased protein stability\(^{(26,27)}\). Further studies would be required to clarify induction mechanism of GAP-43 in DRG sensory neurons.

Erk1/2, along with p38 and JNK proteins is a mitogen-activated protein (MAP) kinase\(^{(36)}\). Erk1/2 is activated by phosphorylation by upstream kinase MEK1/2 protein kinase and in turn, phosphorylates the substrate proteins such as cAMP responsive element binding protein (CREB). Erk1/2 has a diverse function in different types of cells, but in general, is important for cell survival, differentiation, and proliferation. While Erk1/2 has been implicated to be upregulated in regenerating axons\(^{(39)}\), their precise role is not known. Here, phospho-Erk1/2 levels were strongly increased in the injured nerve and further upregulated by SMS treatment. Phospho-Erk1/2 was increased also in the DRG after sciatic neve injury, suggesting retrograde transport of phospho-Erk1/2 into the neuronal cell body area, as has been
implicated in the previous study\textsuperscript{30}. SMS did not alter protein levels in the DRG implying that local control of phospho-Erk1/2 protein may be more important for SMS-mediated axonal regeneration.

Based on the current investigation, several experimental approaches to examine whether SMS is effective as a possible regulator activating regenerative responses appear to be feasible for axonal regeneration. However, the use of SMS is accompanied by difficulties in controlling the quantitative aspect of the treatment because of insufficient knowledge about which component(s) is ultimately active and how the component-component interaction affects the overall action. Future studies at molecular levels using specific chemical components from SMS may provide an insight into establishing therapeutic strategies for the treatment of injured spinal cord.

References
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