



Intravenous Infusion of Ginsenoside Rb1 Ameliorates Compressive Spinal Cord Injury through Upregulation of Bcl-x_L and VEGF

Pengxiang Zhu¹, Ryuji Hata^{2*}, Kimihiko Nakata¹, Fang Cao¹, Keiichi Samukawa¹, Hiroko Fujita¹ and Masahiro Sakanaka¹

¹Department of Functional Histology, Ehime University Graduate School of Medicine, Japan

²Department of Anatomy 1, Fujita Health University School of Medicine, Japan

*Corresponding author: Ryuji Hata, Department of Anatomy 1, Fujita Health University School of Medicine, Toyoake, Aichi, 490-1192, Japan, Tel: +81-562-93-2444, Fax: +81-562-93-5903, E-mail: hata@fujita-hu.ac.jp

Abstract

Background: Red ginseng root (Panax Ginseng C.A. Meyer) has been used clinically by many Asian people for thousands of years without any detrimental effects. One of the major components of red ginseng root is ginsenoside Rb₁ (gRb₁, C₅₄H₉₂O₂₃, molecular weight 1109.46). Previously, we showed that intravenous infusion of gRb₁ attenuated ischemic brain damage in rats through up-regulation of an anti-apoptotic factor, Bcl-x_L. In the present study, we have investigated whether intravenous infusion of gRb₁ attenuates spinal cord injury or not.

Methods: Adult male Wistar rats aged 12-14 weeks, weighing 250-300g, were used. Spinal cord injury (SCI) was induced by compressing the thoracic cord (Th12) with a 20 g weight for 20 minutes. Thirty minutes later, a total of 60μl gRb₁ solution (12 or 60μg/60μl in saline) or the same volume of vehicle (saline) was infused once into the left femoral vein. Subsequently, continuous intravenous administration of gRb₁ was performed for 7 days. The locomotor function was assessed using the Basso-Beattie-Bresnahan (BBB) score and the open field test at 1, 3 or 7 days after SCI. The animals were killed at 7 days after SCI and the SCI lesions were assessed histologically.

Results: Post-traumatic intravenous infusion of gRb₁ (12μg/day or 60μg/day) ameliorated BBB score, rearing activity and neuronal density in the anterior horn of the damaged spinal cord at 7 days after SCI. Subsequent studies that focused on gRb₁-induced expression of gene products responsible for neuronal death or survival revealed that gRb₁ upregulated the expression of not only Bcl-x_L, but also a potent angiogenic and neurotrophic factor, vascular endothelial growth factor (VEGF) at 7 days after SCI.

Conclusion: Ginsenoside Rb₁ (gRb₁), with putative anti-apoptotic, angiogenic and neurotrophic actions, is a candidate agent for the treatment of patients with acute SCI.

Keywords

Ginsenoside Rb₁, Spinal cord injury, Bcl-x_L, VEGF, Apoptosis

Abbreviations

SCI: Spinal Cord Injury, iPS cells: Induced Pluripotent Stem Cells, ES cells: Embryonic Stem Cells, gRb₁: Ginsenoside Rb₁, Bcl-x_L: B-cell Lymphoma-Extra Large, VEGF: Vascular Endothelial Growth Factor

Introduction

The annual incidence of spinal cord injury (SCI) has been estimated to be approximately 20-40 persons per million in the world [1]. The main causes of trauma to the cord are motor-vehicle accidents, sports and recreational activities, work-related accidents and falls at home. More importantly, the majority of SCI victims is young and otherwise healthy, and suffers the burden of life-long disability. There is no universally accepted treatment for this pathological condition, although intensive efforts are underway to develop effective therapy for SCI (for example chondroitinase ABC, Nogo inhibitor, Rho inhibitor, and cell therapy with neural stem/progenitor cells derived from iPS/ES cells [2,3]). At present, administration of methylprednisolone, surgical decompression of the spinal cord, and stabilization of the vertebrae are performed to prevent further injury. However, recovery from functional loss is limited [4,5].

Over the past several decades, researchers have been making desperate efforts, without any great success, searching for intravenously infusible neuroprotective agents for SCI. At present, only methylprednisolone is clinically available. However this agent, even though alleviating SCI, frequently causes serious adverse effects in humans by modulating inflammatory responses in an adverse manner [6]. Finding an intravenously infusible neuroprotective agent that acts safely and favorably on the damaged spinal cord tissue without an increased risk of infection or avascular necrosis of the femoral head appears to be a prerequisite for the treatment of SCI. We speculate that development of an intravenously infusible agent with a potent neuroprotective action would be of great value from the clinical point of view, because such an agent would greatly contribute to elucidation of the molecular mechanisms underlying SCI for which the agent is applied, and would facilitate the development of other innovative treatment protocols and drugs.

Red ginseng root (Panax ginseng C.A. Meyer) is used clinically in Asian countries for various diseases, including atherosclerosis, liver dysfunction, cerebrovascular disease, hypertension and postmenopausal disorders. Ginseng root consists of two major ingredients: crude ginseng saponin and crude ginseng non-saponin fractions. To date, more than 20 saponins have been isolated from

Citation: Zhu P, Hata R, Nakata K, Cao F, Samukawa K, et al., (2015) Intravenous Infusion of Ginsenoside Rb1 Ameliorates Compressive Spinal Cord Injury through Upregulation of Bcl-x_L and VEGF. Int J Neurol Neurother 1:017. doi.org/10.23937/2378-3001/2/1/1017

Received: November 21, 2014; **Accepted:** January 13, 2015; **Published:** January 15, 2015

Copyright: © 2015 Zhu P. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

ginseng root and identified chemically. They can be classified into three major groups according to their chemical structure: protopanaxadiol, protopanaxatriol and oleanolic acid saponins, of which ginsenoside Rb₁ (gRb1), ginsenoside Rg₁ and ginsenoside R₀ are respective representative substances [7].

In our previous study, we showed that postischemic intravenous infusion of gRb1 ameliorated cortical infarct size, place navigation disability and secondary thalamic degeneration after permanent occlusion of the middle cerebral artery (MCA) in stroke-prone spontaneously hypertensive rats (SHR-SP), and demonstrated that gRb1 upregulated the expression of an anti-apoptotic factor, neuronal Bcl-x_L, and prevented neuronal apoptosis *in vitro* and *in vivo* [8]. The potent neuroprotective action of gRb1 in cases of brain ischemia prompted us to investigate the effects of gRb1 on spinal cord injury, since a chemical derivative of gRb1 is known to alleviate the neuroparalysis of rats with SCI [9]. In the present study, we first showed that intravenous infusion of gRb1 ameliorated SCI in rats. Subsequent studies that focused on gRb1-induced expression of gene products responsible for neuronal death or survival revealed that gRb1 upregulated the expression of not only Bcl-x_L, but also a potent angiogenic and neurotrophic factor, VEGF.

Materials and Methods

Adult male Wistar rats aged 12-14 weeks, weighing 250-300 g, were used. Animals were housed in an animal room with a temperature range of 21 to 23°C and a 12-hour light/dark cycle (light on: 7 a.m. to 7 p.m.), with access to food and water *ad libitum* until the end of the experiment.

Production and purification of ginsenoside Rb1 (gRb1)

Ginsenoside Rb1 (gRb1) was isolated and purified from the crude saponin fraction of the rhizome of *Panax Ginseng* C.A. Meyer by repeated column chromatography on silica gel with CHCl₃-MeOH-H₂O (65:35:10) and on ODS (octadecylsilyl) silica with MeOH-H₂O (1:1 - 7:3) [10].

Osmotic minipump implantation

An osmotic minipump (Model 2001, Alza Corp., Palo Alto, CA, USA) filled with either gRb1 solutions (100µg/ 200µl in saline or 500µg/200µl in saline) or vehicle (saline only) was implanted subcutaneously in the back of each animal before traumatic insult, and a fine silicon tube connected to the mini pump was inserted into the left femoral vein after traumatic insult.

Spinal cord injury (SCI) in Wistar rat

Adult male Wistar rats aged 12-14 weeks, weighing 250-300g, were randomly divided into four groups: sham-operated, control, gRb1(12µg/day and 60µg/day)-treated groups. The doses of gRb1 were determined on the basis of our previous study [8] which dealt with the therapeutic effects of intravenously infused gRb1 on middle cerebral artery (MCA)-occluded rats. Rats were anesthetized with 1.5% halothane in a 4:3 mixture of nitrous oxide and oxygen, and body temperature was kept at 37.0±0.2°C during surgery. Spinal cord injury (SCI) was induced as described elsewhere [11]. In brief, the back of the rat was shaved and disinfected. A longitudinal incision was made from the mid to low thoracic vertebrae. The dorsal surface of the spinal cord was exposed by laminectomy of the lower thoracic cord (Th12) vertebrae, and the dura was left intact. The exposed Th12 was compressed extradurally with a 20 g weight for 20 minutes. The muscles and skin were sutured with 4-0 silk. This method induces temporary paralysis of the lower extremities in a reproducible manner [12]. Thirty minutes later, a total of 60 µl gRb1 solution (12 or 60 µg/60 µl in saline) or the same volume of vehicle (saline) was infused once into the left femoral vein. Subsequently, continuous intravenous administration of gRb1 was performed for 7 days via an osmotic minipump (12 or 60 µg/day). Sham-operated animals were administered the same volume of physiological saline (vehicle).

Behavioral evaluation after SCI

As an open field test, a transparent acrylic box (30 x 30 x 30 cm) covered with a sound-attenuating shell (BOF-102, Biomedica, Osaka,

Japan) was used. Inside the open field, an overhead incandescent bulb provided room lighting (approximately 110 lux), and a fan attached to the upper part of the box provided a masking noise of 45 dB. Animals were allowed to move freely in the open field for 20 min in the light condition. On each X and Y bank of the open field, infrared beams were attached 2 cm above the floor at 10-cm intervals, making a flip-flop circuit between the beams. The total number of circuit breaks was counted as locomotor activity. On the X bank, 11 infrared beams were attached 12 cm above the floor at 2.5-cm intervals, and the total number of beam crossings was counted as rearing activity. The open field locomotor scores (locomotor activity, rearing activity and Basso-Beattie- Bresnahan (BBB) scores [13]) were measured before the loading of spinal cord injury, just after the loading of SCI, and from 1 day to 7 days after SCI, as indices of motor function. BBB score of sham-operated rats was 21.

Histological observation and semi-quantitative analysis of MAP2, VEGF and Bcl-x_L protein expression after SCI

After evaluating behavioral performance, animals were anesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg) at 7 days after SCI and perfused transcardially with 0.1 M phosphate-buffered saline (pH 7.4) followed by perfusion with 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The damaged spinal cord (1 cm length) was dissected out. After fixation with the same fixative overnight, each tissue sample was dehydrated, embedded in paraffin, and 5µm-thick serial sections were cut. Each deparaffinized tissue section was treated with 10% non-immunized goat serum and incubated with a monoclonal antibody against microtubule-associated protein 2 (MAP2, SMI 52, Sternberger Monoclonals Inc., Lutherville, MD, USA) overnight at 4°C. After incubation with biotinylated anti-mouse IgG and peroxidase-conjugated streptavidin (DAKO A/S, Glostrup, Denmark), staining was visualized with 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide.

Each animal (n=5 in each group) was anesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg) at 7 days after SCI. The damaged spinal cord (1 cm length) was dissected out. The samples were homogenized on ice with lysis buffer (0.5% SDS, 0.5% Triton-X, 100µM phenylmethane sulfonyl fluoride, 20µM Tris-HCl pH 8.0). Then the samples were sonicated and centrifuged at 13,000 rpm for 10 min at 4°C. The protein content in the supernatant was determined using a BCA protein assay kit (Pierce, Rockland, IL, USA). The supernatant was mixed with sample buffer (62.5mM Tris-HCl, pH 6.8, 2% sodium dodecylsulfate, 10% glycerol and 0.001% bromophenol blue) to a final protein concentration of 1 mg/ml. Equal amounts of protein (15 µg) from the homogenates was electrophoresed and processed for immunoblot analysis using mouse monoclonal antibodies against MAP2 (SMI 52, Sternberger Monoclonals Inc.), VEGF (Santa Cruz Biotech., Santa Cruz, CA, USA) and Bcl-x_L (Transduction Laboratories Inc., Lexington, KY, USA). For semi-quantitative evaluation, the immunoreactive bands were subjected to densitometric analysis using an NIH Image program (National Institutes of Health, Bethesda, MD, USA).

The number of MAP2-positive cells was counted in five 0.25mm x0.25 mm fields in the anterior horns of the spinal cord of the rats. The numerical density of MAP2- positive cells was determined in each of 2 coronal sections per animals. The mean number of positive cells per unit area (per mm²) was calculated in all animals and expressed as mean ± SD.

Statistics

All values are presented as mean value ± SD. Statistical significance was tested by one way ANOVA followed by Bonferroni's multiple comparison test. A p value less than 0.05 was considered to be statistically significant.

Results

Intravenous infusion of gRb1 ameliorates motor deficit and behavioral abnormalities after SCI in rats

We first investigated the effects of intravenous infusion of gRb1

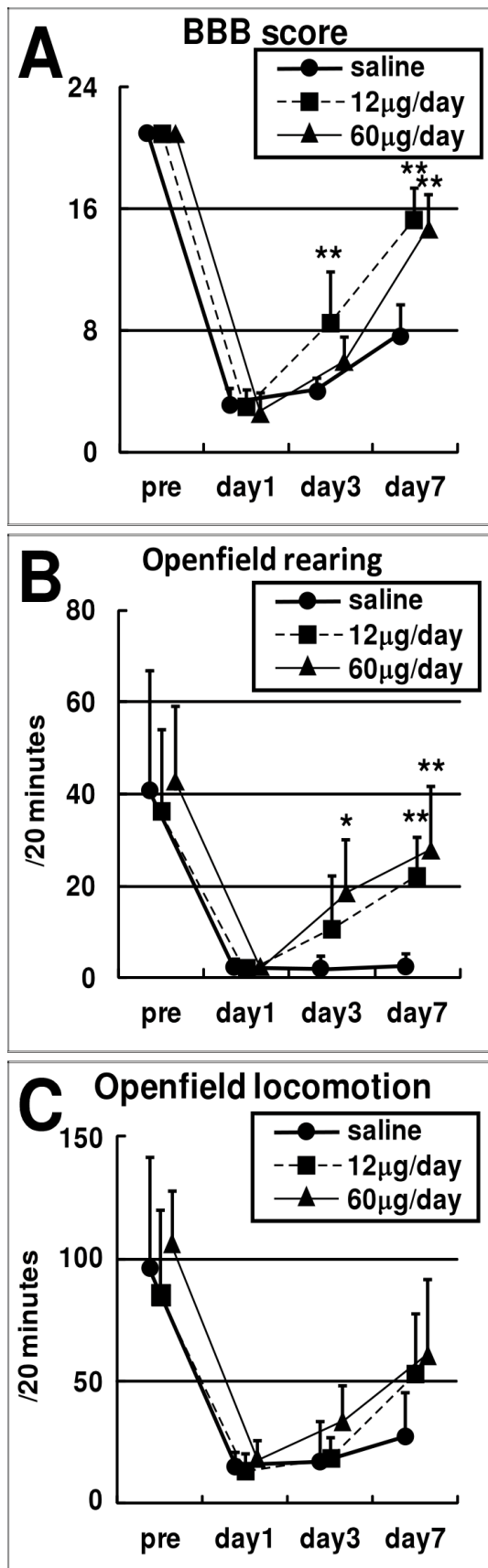


Figure 1: Intravenous infusion of ginsenoside Rb1 (gRb1) improved BBB score and rearing activity after spinal cord injury in Wistar rats.

Note the significant improvement of BBB score and rearing activity in gRb1-treated rats (n = 5 in each group). All values are presented as mean value \pm SD. *, ** indicate significantly higher ($p < 0.05$, $p < 0.01$, respectively) than saline-treated control.

on BBB score (Figure 1A), rearing activity (Figure 1B) and locomotor activity (Figure 1C) in rats. All animals survived until the end of the

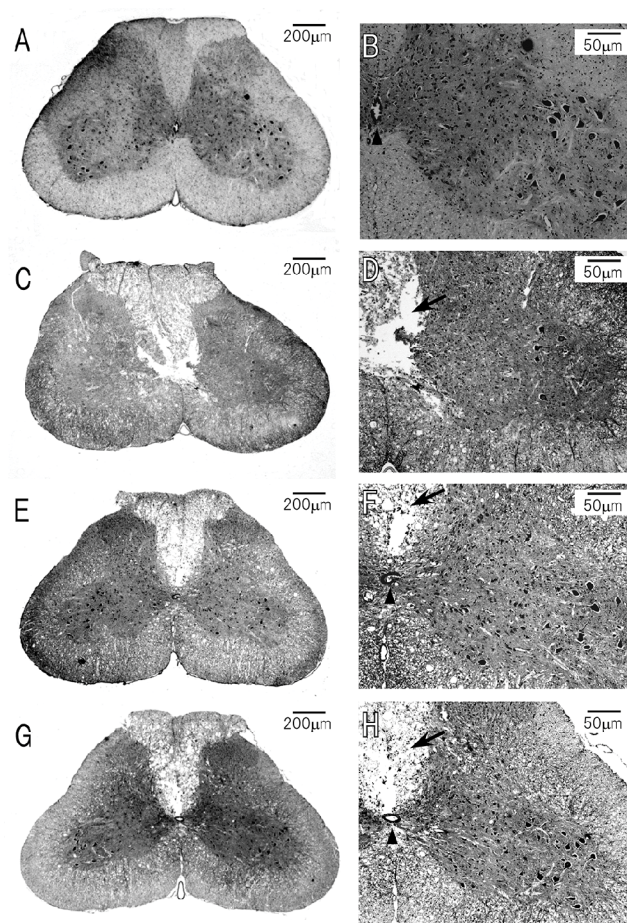


Figure 2: Intravenous infusion of ginsenoside Rb1 (gRb1) ameliorated morphological damage to the spinal cord at 7 days after spinal cord injury (SCI) in Wistar rats.

A-H: Representative photomicrographs of MAP2 immunostaining in sections from spinal cord of sham-operated rat and damaged spinal cords of rats at 7 days after SCI (A and B: Sham; C and D: saline; E and F: gRb1 (12 µg/day); G and H: gRb1 (60 µg/day)).

Scale bar = 200µm in A, C, E, G and 50 µm in B, D, F, H.

Note cracks in the white matters (D, F, H, arrows) and the central canals (B, F, H, arrowheads).

experiment and exhibited motor deficit and behavioral abnormalities after SCI. Compared with the vehicle-treated group, the gRb1-treated groups showed significant improvement of behavioral abnormalities and rearing activity with respect to BBB score and rearing activity at 7 days after SCI (n = 5 in each group).

Intravenous infusion of gRb1 ameliorates morphological damage to the spinal cord after SCI in rats

To evaluate morphological damage after SCI, we next performed immunostaining for MAP2 (Figure 2A-H). In the sham-operated rat (Figures 2A and B), MAP2 immunoreactivity was localized predominantly to dendrites and neuronal soma within the gray matter and white matter of the spinal cord that was endowed with the central canal (Figure 2B, arrowhead). In the spinal cord of the saline-treated rat at 7 days after SCI (Figures 2C and D), the white matter particularly the bilateral dorsal funiculi had a wide and irregular shaped crack and moreover the central canal and the gray matter adjacent to it were destroyed (Figure 2D, arrow). The intravenous infusion of gRb1 at a dose of 12µg/day (Figures 2E and F) or 60µg/day (Figures 2G and H) after SCI apparently made smaller the crack in the dorsal funiculi, and rescued the gray matter around the central canal as well as the canal per se (Figures 2F and H, arrows and arrowheads). To quantify the neuroal damage caused by SCI, MAP2- positive cells per mm² in each group were counted and the results demonstrated that MAP2-positive cells were significantly more numerous in the gRb1-treated groups than in the saline-infused group after SCI (12

Neuron's number in the anterior horn

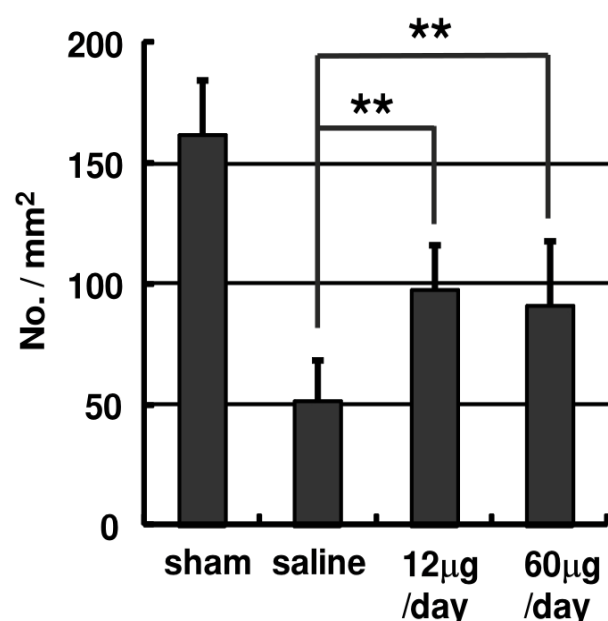


Figure 3: Intravenous infusion of ginsenoside Rb1(gRb1) inhibited loss of MAP2-positive neurons and increased neural density of the anterior horn of damaged spinal cord at 7 days after spinal cord injury (SCI) in Wistar rats.

Note the significant improvement of neural density in gRb1-treated rats (n = 5 in each group).

Data were obtained from five independent experiments. All values are presented as mean \pm SD. ** indicates significantly higher ($p < 0.01$) than saline-treated control.

$\mu\text{g/day}$: $97.6 \pm 18.8/\text{mm}^2$ [mean \pm SD]; $60 \mu\text{g/day}$: $91.3 \pm 26.2/\text{mm}^2$ vs. Saline: $51.3 \pm 17.1/\text{mm}^2$), as shown in Figure 3.

For further quantitative analysis of SCI damage, we conducted western blot using an antibody against MAP2 protein. Compared with the saline-treated group, MAP2 protein expression was clearly up-regulated in the spinal cord obtained from the gRb1-treated groups (Figure 4A). The same experiments were independently performed five times, and densitometric analysis of the immunoreactive bands revealed that, compared with the saline-treated control, treatment with gRb1 at doses of 12 and $60 \mu\text{g/day}$ caused 1.86-fold and 1.88-fold increases in MAP2 protein expression, respectively, in the damaged spinal cord of rats at seven days after SCI (Figure 4B).

Ginsenoside Rb1 (gRb1) upregulates expression of Bcl-x_L and VEGF in spinal cord

To gain an insight into the molecular mechanisms underlying gRb1-mediated neuroprotection, we conducted western blot using an antibody against VEGF or Bcl-x_L protein. VEGF and Bcl-x_L proteins with molecular masses of approximately 26 kD and 29 kD, respectively were constitutively expressed in the spinal cord (Figure 4A). Both VEGF and Bcl-x_L protein expression were clearly upregulated in the injured spinal cord from rats treated with 12 or $60 \mu\text{g/day}$ of gRb1 (Figure 4A), at seven days after SCI. The same experiments were independently performed five times, and densitometric analysis of the immunoreactive bands revealed that treatment with gRb1 at doses of 12 and $60 \mu\text{g/day}$ caused 1.9-fold and 2.1-fold increases in Bcl-x_L protein expression (Figure 4C) and 1.8-fold and 1.8-fold increases in VEGF protein expression (Figure 4D) in the injured spinal cord, at seven days after SCI.

Discussion

Using stroke-prone spontaneously hypertensive rats (SHR-SP rats), we previously demonstrated a protective effect of intravenously

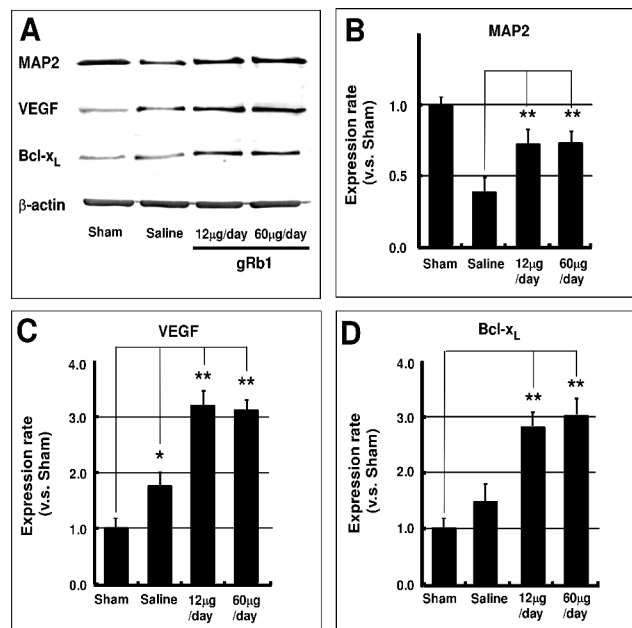


Figure 4: Ginsenoside Rb1(gRb1) reduced neuronal damage detected by MAP2 immunoblot and upregulated both VEGF and Bcl-x_L protein expression in damaged spinal cord at 7 days after spinal cord injury (SCI) in Wistar rats.

A: Representative photographs of Western blot for MAP2, VEGF and Bcl-x_L protein in rat damaged spinal cord at 7 days after SCI. β -actin was used as an internal control for each sample.

B-D: Densitometric analysis of MAP2 (B), VEGF (C) and Bcl-x_L (D)-immunoreactive bands in rat damaged spinal cord at 7 days after SCI.

B: Densitometric analysis of MAP2-immunoreactive bands revealed that MAP2 protein expression in damaged spinal cord from ginsenoside Rb1 (gRb1)-treated rats was significantly improved, compared with saline-treated control.

C and D: Note that VEGF and Bcl-x_L protein expression were upregulated by treatment with gRb1.

Data were obtained from five independent experiments. All values are presented as mean \pm SD. **, * indicate significantly higher ($p < 0.01$, $p < 0.05$) than sham (C and D) or saline-treated control (B).

administered gRb1 against brain damage after cerebral infarction through the upregulation of Bcl-x_L, both *in vivo* and *in vitro* [8]. We also demonstrated the protective effect of dihydroginsenoside Rb1 (dgRb1) at lower doses against brain damage after cerebral infarction through the up-regulation of VEGF and Bcl-x_L [9]. DgRb1 is a stable and novel chemical derivative of gRb1 and the lack of double bond in the chemical structure made dgRb1 more stable, and harder to be metabolized compared with gRb1. This may account for why the effective dose of intravenously infused dgRb1 is one tenth of that of Rb1 for the treatment of cerebral infarction. Taken the present experimental results and the former experimental findings all together, the effective dose of dgRb1 for the therapy of SCI is also one tenth of that of gRb1 [9]. Even though the pharmacological property of dgRb1 is superior to that of gRb1, we would recommend the application of gRb1 rather than dgRb1 for the treatment of cerebral stroke and neurotrauma, keeping in mind that humans have taken ginseng containing gRb1 for approximately 2000 years without any serious adverse effects.

The pathological sequelae after SCI are divided into two broad chronological events: primary injury and secondary injury [14,15]. Primary injury is caused by direct mechanical trauma to the spinal cord where focal destruction occurs. This initial injury is followed by secondary injury. A series of processes in secondary injury including ischemia, edema, revascularization and inflammation are thought to enlarge the area of cell death through necrosis, apoptosis and autophagy [14,16,17]. Among them, ischemia has been demonstrated to be a crucial factor in post-injury pathophysiological changes in acute SCI, because it is believed to aggravate secondary injury and to occur in parallel to neurological dysfunction.

Revascularization is, therefore, one of the most important factors to regain blood flow in the ischemic tissue. Secondary injury of the spinal cord declined along with revascularization of the involved tissue [18]. This evidence suggests that revascularization precedes spinal tissue repair and nerve regeneration, and may ameliorate the cascade of progressive cell death. In our previous study, we showed that gRb1 reorganized the cerebrovascular networks in the ischemic penumbra, suggesting that gRb1 could induce proliferation of endothelial cells and stimulate angiogenesis [8]. Vascular endothelial growth factor (VEGF) is considered to be a potent endothelial cell-specific growth factor [19], and gRb1 has been shown to increase VEGF expression in keratinocytes during the wound-healing process [20]. Hence, we evaluated whether gRb1 upregulated VEGF expression in the damaged spinal cord, and showed that it did.

VEGF was initially described in 1983 as a 'tumor-secreted vascular permeability factor' [21] and was cloned in 1989 [22]. VEGF has been established as an essential regulator of angiogenesis in a variety of human diseases [23,24]. Although initial studies indicated that VEGF is an endothelial cell-specific factor, more recent findings revealed that VEGF also has direct effects on neural cells. In the dorsal root ganglia, VEGF stimulates axonal outgrowth and promotes the survival of neurons and satellite cells, whereas inhibition of VEGF receptor-2 (VEGFR-2) signaling blocks axonal outgrowth in response to VEGF [25]. VEGF also has neurotrophic effects on cultured neurons of the central nervous system and, in many instances, exerts this effect via signaling through VEGFR-2, phosphatidylinositol-3-kinase (PI3K) and Akt [19]. The ability of VEGF to stimulate angiogenesis and elicit direct neurotrophic effects makes it an attractive candidate for promoting repair or regeneration of damaged brain tissue [26].

In a model of cerebral ischemia, upregulation of VEGF was detected in the penumbra, where neovascularization and neuronal apoptosis of CNS are known to occur [27,28]. However, VEGF up-regulation following brain damage may have deleterious effects due to its permeability-increasing activity *in vivo*. Following SCI, treatment with recombinant VEGF caused an improvement in recovery, associated with increased vessel density and reduced apoptosis, in the lesion area, and increased expression of VEGFR1, VEGFR2, NP1 and NP2 [29], while it was also found to have a worsening effect on lesions caused by SCI, possibly secondary to its effect on vascular permeability [30]. The timing of VEGF treatment is probably an important determinant of its therapeutic effect on brain damage. Early treatment with VEGF (1 h post-ischemia) increased blood-brain barrier leakage, hemorrhagic transformation and ischemic lesions, while late treatment with VEGF (48 h post-ischemia) enhanced angiogenesis and significantly improved neurological recovery [31]. It is plausible that the intravenous infusion of gRb1 after SCI caused a favorable effect similar to that of the aforementioned late treatment with VEGF by way of the up-regulation of VEGF expression. There is also a report showing VEGFR2 expression in the choroid plexus and ependymal cells [32]. This may provide a molecular basis for the gRb1-mediated rescue of the central canal that is encircled by ependymal cells. In summary, we speculate that the neuroprotective effects of VEGF result from a combination of direct effects on neuronal survival as well as indirect consequences of increased angiogenesis, and that spontaneous late-onset upregulation by gRb1 of VEGF protein in neurons can ameliorate SCI and ischemic brain damage *in vivo*. Further investigations are required to confirm this assumption.

Apoptosis and autophagy are also important factors that promote cell death and aggravate secondary injury. In this study, we have showed that gRb1 upregulated Bcl-x_L, an anti-apoptotic Bcl-2 family member, in the damaged spinal cord, as was so in our previous study concerning cerebral infarction [8]. Bcl-x_L, as a mitochondrion-associated protein, is widely expressed in the nervous system [33,34]. Mice deficient in Bcl-x_L die on embryonic day 13 (E13), and analysis of E12 mouse embryos showed massive death of immature postmitotic neurons throughout the brain and spinal cord as well as in the developing dorsal root ganglia [35]. Moreover, overexpression

of Bcl-x_L leads to increased survival of postnatal central neurons [36]. In the pathological mechanism of SCI and ischemic brain damage, insufficient expression of Bcl-x_L protein in response to SCI and brain damage appears to liberate Apaf1 and cytochrome c, which form a complex with procaspase 9, leading to activation of procaspase 9 and caspase 9, and then to activation of the cell executioner, caspase 3 [37]. Moreover, Bcl-x_L binds to Beclin 1 and inhibits Beclin 1-mediated autophagy. Bcl-x_L and Beclin 1 also cooperate with Atg5 or Ca²⁺ to regulate both apoptosis and autophagy [38]. Consistent with these previous studies, our data have suggested that administration of gRb1 ameliorated SCI, at least in part, through the upregulation of an anti-apoptotic factor, Bcl-x_L.

To our best knowledge, *in vitro* studies that demonstrate gRb1-mediated upregulation of Bcl-x_L and VEGF in spinal neurons have not been published yet. This raises the question of whether or not intravenously infused gRb1 acted directly on spinal cord neurons in the present *in vivo* study. In our previous *in vitro* experiments, gRb1 upregulated the expressions of bcl-x_L mRNA and Bcl-x_L protein in cortical neurons, and subsequent promoter assays with the use of bcl-xl-wt carrying the stat 5 response element (STRE), bcl-xl-mut having a mutated STRE and bcl-xl-0.6L devoid of STRE indicated that the STRE on the bcl-xl promoter is responsible for the induction of bcl-xl mRNA by gRb1 in cultured cortical neurons [8]. Although there are no reports showing gRb1-mediated upregulation of VEGF in cultured neurons, [20] demonstrated that gRb1 stimulates VEGF expression in cultured HaCaT cells in the presence of interleukin-1β (IL-1β). Moreover, dihydroginsenoside Rb1 (dgRb1), a chemical derivative of gRb1, with *in vivo* neuroprotective actions similar to those of gRb1 in cases of brain ischemia and compressive spinal cord injury is also known to facilitate the expressions of VEGF mRNA, VEGF protein, bcl-x_L mRNA and Bcl-x_L protein in cultured cortical neurons and to induce VEGF mRNA and bcl-x_L mRNA in hypoxia response element (HRE) - and STRE- dependent manners, respectively, in the cortical neurons [9]. These findings are in favor of the notion that intravenously administered gRb1 acts directly on spinal cord neurons to stimulate the expressions of Bcl-x_L and VEGF, thereby protecting the neurons against spinal cord injury. To ascertain further the neuroprotective action of gRb1 by way of Bcl-xl and VEGF *in vitro*, knock down experiments using siRNA would be of help.

Conclusions

The results of this study demonstrated that ginsenoside Rb1 (gRb1), one of the major components of red ginseng root (Panax Ginseng C.A. Meyer), ameliorates SCI in rats by up-regulation of a potent angiogenic and neurotrophic factor, VEGF as well as an anti-apoptotic and anti-autophagic factor, Bcl-x_L.

These results suggest that gRb1 is a candidate agent for the treatment of patients with acute SCI.

Acknowledgments

This research project was supported, in part, by grants from the Ministry of Education, Science, Sports and Culture of Japan and from the Japan Red Ginseng Research Association. The secretarial assistance of Ms. K. Hiraoka is acknowledged.

Ethical Statement

All experiments were approved by the Ethics Committee of Ehime University School of Medicine and were conducted according to the Guidelines for Animal Experimentation at Ehime University School of Medicine.

References

1. Cadotte DW, Fehlings MG (2011) Spinal cord injury: a systematic review of current treatment options. *Clin Orthop Relat Res* 469: 732-741.
2. Kwon BK, Okon EB, Plunet W, Baptiste D, Fouad K, et al. (2011) A systematic review of directly applied biologic therapies for acute spinal cord injury. *J Neurotrauma* 28: 1589-1610.
3. Tsuji O, Miura K, Fujiyoshi K, Momoshima S, Nakamura M, et al. (2011) Cell therapy for spinal cord injury by neural stem/progenitor cells derived from iPS/ES cells. *Neurotherapeutics* 8: 668-676.

4. Enomoto M, Wakabayashi Y, Qi ML, Shinomiya K (2004) Present situation and future aspects of spinal cord regeneration. *J Orthop Sci* 9: 108-112.
5. Rabchevsky AG, Patel SP, Springer JE (2011) Pharmacological interventions for spinal cord injury: where do we stand? How might we step forward?. *Pharmacol Ther* 132: 15-29.
6. Sayer FT, Kronvall E, Nilsson OG (2006) Methylprednisolone treatment in acute spinal cord injury: the myth challenged through a structured analysis of published literature. *Spine J* 6: 335-343.
7. Shibata S, Tanaka O, Shoji J, Saito H (1994) Chemistry and pharmacology of Panax. In: Wagner H, Hikino H, Earnsworth RN (eds) *Economic and medicinal plant research*. World Scientific Philadelphia, pp 217-284.
8. Zhang B, Hata R, Zhu P, Sato K, Wen TC, et al. (2006) Prevention of ischemic neuronal death by intravenous infusion of a ginseng saponin, ginsenoside Rb(1), that upregulates Bcl-x(L) expression. *J Cereb Blood Flow Metab* 26: 708-721.
9. Sakanaka M, Zhu P, Zhang B, Wen TC, Cao F, et al. (2007) Intravenous infusion of dihydroginsenoside Rb1 prevents compressive spinal cord injury and ischemic brain damage through upregulation of VEGF and Bcl-XL. *J Neurotrauma* 24: 1037-1054.
10. Samukawa H, Yamashita H, Matsuda H, Kubo M (1995) Simultaneous analysis of saponins in Ginseng Radix by high performance liquid chromatography. *Chem Pharm Bull* 43:137-141.
11. Morino T, Ogata T, Horiuchi H, Takeba J, Okumura H, et al. (2003) Delayed neuronal damage related to microglia proliferation after mild spinal cord compression injury. *Neurosci Res* 46: 309-318.
12. Naruo S, Okajima K, Taoka Y, Uchiba M, Nakamura T, et al. (2003) Prostaglandin E1 reduces compression trauma-induced spinal cord injury in rats mainly by inhibiting neutrophil activation. *J Neurotrauma* 20: 221-228.
13. Basso DM, Beattie MS, Bresnahan JC (1995) A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 12: 1-21.
14. Oyinbo CA (2011) Secondary injury mechanisms in traumatic spinal cord injury: a nugget of this multiply cascade. *Acta Neurobiol Exp (Wars)* 71: 281-299.
15. Profyris C, Cheema SS, Zang D, Azari MF, Boyle K, et al. (2004) Degenerative and regenerative mechanisms governing spinal cord injury. *Neurobiol Dis* 15: 415-436.
16. Beattie MS (2004) Inflammation and apoptosis: linked therapeutic targets in spinal cord injury. *Trends Mol Med* 10: 580-583.
17. Kanno H, Ozawa H, Sekiguchi A, Itoi E (2009) Spinal cord injury induces upregulation of Beclin 1 and promotes autophagic cell death. *Neurobiol Dis* 33: 143-148.
18. Zhang Z, Guth L (1997) Experimental spinal cord injury: Wallerian degeneration in the dorsal column is followed by revascularization, glial proliferation, and nerve regeneration. *Exp Neurol* 147: 159-171.
19. Storkebaum E, Lambrechts D, Carmeliet P (2004) VEGF: once regarded as a specific angiogenic factor, now implicated in neuroprotection. *Bioessays* 26: 943-954.
20. Kimura Y, Sumiyoshi M, Kawahira K, Sakanaka M (2006) Effects of ginseng saponins isolated from Red Ginseng roots on burn wound healing in mice. *Br J Pharmacol* 148: 860-870.
21. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, et al. (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 219: 983-985.
22. Ferrara N, Henzel WJ (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 161: 851-858.
23. Eichmann A, Simons M (2012) VEGF signaling inside vascular endothelial cells and beyond. *Curr Opin Cell Biol* 24: 188-193.
24. Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. *Nat Med* 9: 669-676.
25. Sondell M, Sundler F, Kanje M (2000) Vascular endothelial growth factor is a neurotrophic factor which stimulates axonal outgrowth through the flk-1 receptor. *Eur J Neurosci* 12: 4243-4254.
26. Mackenzie F, Ruhrberg C (2012) Diverse roles for VEGF-A in the nervous system. *Development* 139: 1371-1380.
27. Ferrer I1, Planas AM (2003) Signaling of cell death and cell survival following focal cerebral ischemia: life and death struggle in the penumbra. See comment in PubMed Commons below *J Neuropathol Exp Neurol* 62: 329-339.
28. Marti HJ, Bernaudin M, Bellail A, Schoch H, Euler M, et al. (2000) Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. *Am J Pathol* 156: 965-976.
29. Widenfalk J, Lipson A, Jubran M, Hofstetter C, Ebendal T, et al. (2003) Vascular endothelial growth factor improves functional outcome and decreases secondary degeneration in experimental spinal cord contusion injury. *Neuroscience* 120: 951-960.
30. Benton RL, Whittemore SR (2003) VEGF165 therapy exacerbates secondary damage following spinal cord injury. *Neurochem Res* 28: 1693-1703.
31. Zhang ZG, Zhang L, Jiang Q, Zhang R, Davies K, et al. (2000) VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. *J Clin Invest* 106: 829-838.
32. Maharaj AS, Walshe TE, Saint-Geniez M, Venkatesha S, Maldonado AE, et al. (2008) VEGF and TGF-beta are required for the maintenance of the choroid plexus and ependyma. *J Exp Med* 205: 491-501.
33. Raghupathi R (2004) Cell death mechanisms following traumatic brain injury. *Brain Pathol* 14: 215-222.
34. Sprick MR, Walczak H (2004) The interplay between the Bcl-2 family and death receptor-mediated apoptosis. *Biochim Biophys Acta* 1644: 125-132.
35. Motoyama N, Wang F, Roth KA, Sawa H, Nakayama K, et al. (1995) Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science* 267: 1506-1510.
36. Parsadanian AS, Cheng Y, Keller-Peck CR, Holtzman DM, Snider WD (1998) Bcl-xL is an antiapoptotic regulator for postnatal CNS neurons. *J Neurosci* 18: 1009-1019.
37. Ouyang YB, Giffard RG (2004) Cellular neuroprotective mechanisms in cerebral ischemia: Bcl-2 family proteins and protection of mitochondrial function. *Cell Calcium* 36: 303-311.
38. Zhou F, Yang Y, Xing D (2011) Bcl-2 and Bcl-xL play important roles in the crosstalk between autophagy and apoptosis. *FEBS J* 278: 403-413.