Inhibitory Effects of *Eleutherococcus senticosus* Extracts on Amyloid β(25-35)–Induced Neuritic Atrophy and Synaptic Loss

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Received February 20, 2008; Accepted May 23, 2008

**Abstract.** Neurons with atrophic neurites may remain alive and therefore may have the potential to regenerate even when neuronal death has occurred in some parts of the brain. This study aimed to explore effects of drugs that can facilitate the regeneration of neurites and the reconstruction of synapses even in severely damaged neurons. We investigated the effects of *Eleutherococcus senticosus* extracts on the regeneration of neurites and the reconstruction of synapses in rat cultured cortical neurons damaged by amyloid β (Aβ)(25-35). Treatment with Aβ(25-35) (10 μM) induced axonal and dendritic atrophies and synaptic loss in cortical neurons. Subsequent treatment with the methanol extract and the water extract of *E. senticosus* (10–1000 ng/ml) resulted in significant axonal and dendritic regenerations and reconstruction of neuronal synapses. Co-application of the extract and Aβ(25-35) attenuated Aβ(25-35)-induced neuronal death. We investigated neurite outgrowth activities of eleutherosides B and E and isoflaxidin, which are known as major compounds in *E. senticosus*. Although eleutheroside B protected against Aβ(25-35)-induced dendritic and axonal atrophies, the activities of eleutheroside E and isofraxidin were less than that of eleutheroside B. Although the contents of these three compounds in the water extract were less than in the methanol extract, restoring activities against neuronal damages were not different between the two extracts. In conclusion, extracts of *E. senticosus* protect against neuritic atrophy and cell death under Aβ treatment, and one of active constituents may be eleutheroside B.

**Keywords:** axon, dendrite, synapse, neuronal death, *Eleutherococcus senticosus*

**Introduction**

In addition to the death of neurons, atrophy of neurites and loss of synapses are the major causes of dysfunctions of the brain in Alzheimer’s disease (1–3). Neurons with atrophic neurites may remain alive and therefore may have the potential to regenerate even when neuronal death has occurred in some parts of the brain. We therefore hypothesized that reconstructing neuronal networks in the injured brain is essential for the recovery of brain function (4). To reconstruct neuronal networks, neurites must be regenerated and synapses must be reconstructed. In the current studies, we explored the in vitro effects of drugs that can facilitate the regeneration of neurites and the reconstruction of synapses even in severely damaged neurons.

Roots of *Eleutherococcus senticosus* RUPR. et MAXIM. (=*Acanthopanax senticosus* HARMS) are used mainly as a tonic and anti-stress drug in traditional Chinese medicine (5). A few reports showed that the water extract of *E. senticosus* affected brain function. For example, the water extract reduces infarct volume in transient focal cerebral ischemia in rats (6). Also, the water extract protects against MPTP-induced cell death in rat brain (7). In our previous studies, herbal medicines used as tonic drugs showed marked improvements against neuritic atrophy, synaptic loss, and memory deficit (8–11). Therefore, we focused on *E. senticosus* as a candidate for anti-dementia drug.

Amyloid β (Aβ) is thought to be a major pathological...
cause of Alzheimer’s disease. Aβ(25-35) is a partial fragment of the full peptide of Aβ and can be produced in Alzheimer’s disease patients by enzymatic cleavage of the naturally occurring Aβ(1-40) (12). Aβ(25-35) similarly forms a β-sheet structure (13) and induces neuronal cell death (13, 14), neurite atrophy (8, 15), synaptic loss (8, 15, 16), and memory impairment (8, 9, 16, 17). Moreover, our previous work also demonstrated that Aβ(25-35) and Aβ(1-42) resulted in similar effects on neuritic atrophy and cell death at 10 μM (10). Furthermore, a recent report showed that a single intracerebroventricular (i.c.v., 15 μg) injection of Aβ(25-35) could induce major neuropathological signs related to early stages of Alzheimer’s disease in rats (18). Considering those reports, we have mainly used Aβ(25-35).

Our previous studies suggested that compounds that showed synaptic regeneration activity in Aβ(25-35)-treated cultured neurons were also active for memory impairment in Alzheimer’s disease model mice (9 – 11). Up until now, effects of E. senticosus on atrophy of neurites and synaptic loss have never been investigated. Therefore, we performed this study to characterize the activities of E. senticosus under Aβ-induced neuritic damage.

**Materials and Methods**

**Materials**

Aβ(25-35) (Sigma, St. Louis, MO, USA) was dissolved in sterile distilled water at a concentration of 5 mM and were incubated at 37°C for 4 days to allow fibril formation. Neurobasal media and B-27 supplement were purchased from Gibco BRL (Rockville, MD, USA). Mouse β-nerve growth factor (NGF) was purchased from Astral Biologicals (San Ramon, CA, USA). A monoclonal antibody (clone SMI-312) to phosphorylated neurofilament-H (NF-H) was purchased from Sternberger Monoclonals, Inc. (Lutherville, MD, USA). A polyclonal antibody and a monoclonal antibody to microtubule-associated protein 2a and 2b (MAP2) were purchased from Chemicon (Temecula, CA, USA). Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 546-conjugated goat anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR, USA). Eleutheroside B and isofraxidin were purchased from Matsuura Yakugyo (Nagoya). Eleutheroside E was purchased from Phytolab (Hamburg, Germany).

**Preparation of extracts**

The methanol extract and water extract of Eleutherococcus senticosus Rhizome (production area: Heilonjang Prov., China; purchased from Tochimoto Tenkaido Co. Ltd., Osaka) were prepared as follows: Thirty grams of grained drug were extracted two times with 300 ml of methanol or water under reflux condition. The combined solvents were evaporated in vacuo to give 1.27 g of methanol extract and 1.40 g of water extract. The methanol extract and water extract were dissolved in dimethyl sulfoxide (DMSO) and sterilized water, respectively. A voucher specimen of Eleutherococcus senticosus Rhizome was deposited with TMPW number 22875 in the Museum of Materia Medica, Research Center for Ethnomedicine, Institute of Natural Medicine.

**Isolation of compounds**

Eleutherococcus senticosus Rhizome (1.0 kg; produced in Heilonjang Prov., purchased from Tochimoto Tenkaido Co., Ltd., and with a voucher number TMPW No. 24214) was extracted with methanol (MeOH) 5 times under reflux condition. The MeOH extract was suspended with water and then extracted with n-hexane. The water layer was extracted with EtOAc to obtain the EtOAc extract (12.02 g). The water layer was further extracted with n-BuOH to obtain the n-BuOH extract (13.38 g) and H2O extract (29.85 g). The EtOAc extract was fractioned by silica gel column, which was eluted with CHCl3-MeOH (20:1) to give fractions 1 – 18. Fractions 7 – 8 were purified using a Sephadex LH-20 column (GE Healthcare, Sweden) and separated by a silica gel column, which was eluted with n-Hexane-Acetone (4:1) to yield isofraxidin (18.5 mg). The n-BuOH extract was fractioned by silica gel column, which was eluted with CHCl3-MeOH (5:1) to give fractions 1 – 5. Fraction 3 was purified using Sephadex LH-20 column, which was eluted with 90% MeOH-H2O to afford fractions 1 – 33. Fraction 25 was evaporated by freeze drying to yield eleutheroside E (21.2 mg). Fractions 28 – 31 were purified using Sephadex LH-20 column, which was eluted with MeOH and followed by a silica gel column, which was eluted with CHCl3-MeOH (5:1) to afford eleutheroside B (32.5 mg). The structures of isofraxidin, eleutheroside E, and eleutheroside B were elucidated by comparing their spectral analyses with those given in the literature.

**Primary culture**

Embryos were removed from pregnant Sprague-Dawley rats (Japan SLC) at 17 – 19 days of gestation. The cortices were dissected and the dura mater was removed. The tissues were minced and dissociated and then grown in cultures with neurobasal medium including 12% horse serum on 8-well chamber slides (Falcon, Franklin Lakes, NJ, USA) coated with poly-D-lysine at 37°C in a humidified incubator with 10% CO2. When Aβ(25-35) or other compounds were added, half of the medium in each well was replaced with fresh medium.
containing 2% B-27 supplement without serum. In cases of long-term culture (for synaptophysin staining), half of the medium in each well was replaced every 7 days with serum-free medium containing the 2% B-27 supplement after initiation of the culture period. The time schedules of the experiments are shown at the bottom of each figure.

**Analysis of neurite outgrowth**

Rat cortical neurons (density: 1.45 × 10⁵ cells/cm²) on 8-well chamber slides were fixed by 4% paraformaldehyde and immunostained with a monoclonal antibody to phosphorylated neurofilament-H (pNF-H) (dilution of 1:1000) as an axonal marker or a monoclonal antibody to MAP2 (dilution 1:1000) as a dendrite marker. Alexa Fluor 488-conjugated goat anti-mouse IgG dilution (1:200) was used as secondary antibodies. The slides were mounted with Aqua Poly Mont. Fluorescence images (four images per treatment) were captured by a fluorescence microscope (AX-80; Olympus, Tokyo) at 421 μm × 322 μm. The length of neurites positive for phosphorylated NF-H or MAP2 was measured by using the image analysis software Neurocyte (Kurabo, Osaka). Total length of neurites (axon or dendrite) in an image was divided by cell numbers in the area. Four areas were measured per treatment.

**Analysis of synaptic formation**

After the drug treatment, the cells were fixed and double-immunostained with a combination of monoclonal antibody to synaptophysin (dilution of 1:500) as a presynaptic marker and a polyclonal antibody to MAP2 (dilution of 1:1000) as a dendrite marker. Alexa Fluor 488–conjugated goat anti-mouse IgG (1:200) and Alexa Fluor 568–conjugated goat anti-rabbit IgG (1:200) were used as secondary antibodies. The slides were mounted with Aqua Poly Mont. The fluorescence images were captured by a confocal laser scanning microscope (Radiance 2100; Bio Rad, Hercules, CA, USA) at 302 μm × 302 μm, and four images were captured per treatment. The area of positive puncta to synaptophysin on each dendrite was measured using an image analyzer ATTO densitograph (ATTO, Tokyo). The length of the dendrites was measured using Scion Image (Scion, Frederick, MD, USA).

**Cell viability**

The cortical culture was treated by 0.4% trypan blue. After 5 min, dead cells stained by trypane blue were counted for evaluation of cell viability.

**Quantifying compounds in extracts**

Eleutherosides B and E or isoflaxidin (10 mg) was accurately weighed into 1-ml volumetric flask and dissolved in methanol. To draw calibration curves, a series of standard solutions were prepared from the stock solution and filtered through a 0.2-μm Millipore filter (Advantec, Tokyo). A 10-μl volume of each of the standards or samples was injected into the HPLC system. The HPLC system (Shimadzu, Kyoto) was composed of an LC-10ADvp HPLC pump, a DGU-14A degaser, CTO-10ASvp column oven, SLC-10ADvp auto sampler, and SPD-M10Avp detector. LC analysis was carried out using a Waters Xterra Phenyl column (4.6 mm i.d. × 150 mm, 3.5 μm) with column temperature at 40°C. The mobile phase was a binary eluent of (A) acetonitrile and (B) 20 mM phosphate buffer (pH 4.0) under flowing gradient conditions: 0 – 5 min linear gradient from 8% to 13% A, 5 – 39 min linear gradient from 13% to 30% A. Flow rate was 0.8 ml/min. Detection wavelength was 264, 207, and 340 nm for eleutheroside B, eleutheroside E, and isoflaxidin, respectively.

**Statistical analysis**

Statistical comparisons were carried out by one-way analysis of variance followed by Dunnett’s post hoc test. Values of P<0.05 were considered significant. The means of the data are presented together with the S.E.M.

**Results**

*Eleutherococcus senticosus* promotes regenerations of axons and dendrites in damaged neurons

We examined the effect of the methanol extract and water extract of *E. senticosus* on neurite regeneration after axonal atrophy had already occurred (8). Therefore, the compounds were administered 3 days after treatment with Aβ(25-35), and axon and dendrite lengths were measured after an additional 5 days. We found that the axon (Fig. 1) and dendrite (Fig. 2) lengths were shorter in the cells treated with Aβ(25-35) than when they were treated with vehicle alone. NGF (100 ng/ml) also enhanced axonal (Fig. 1) and dendritic (Fig. 2) extension when treated after Aβ(25-35). Potencies of the methanol extract and water extract for neuritic regeneration were not clearly different.

**Synaptic reconstruction in damaged neurons**

Determining whether regenerated neurites can also reconstruct synapses is essential. Since the methanol...
Fig. 1. Effects of *Eleutherococcus senticosus* extracts on axons after Aβ(25-35)-induced atrophy. Cortical neurons were cultured for 3 days and then treated with 10 μM Aβ(25-35) or water (Cont). Three days after the administration of Aβ(25-35), cells were treated with the methanol (A) or water (B) extract of *E. senticosus* (10 – 1000 ng/ml) or the vehicle (0.1% DMSO, Veh). Five days after treatment, the cells were fixed and immunostained for phosphorylated NF-H. The lengths of phosphorylated NF-H-positive neurites were measured. Values are means ± S.E.M. of data from 4 areas. Representative photographs are shown with a scale bar. *P<0.05, when compared with Veh.
Fig. 2. Effects of *E. senticosus* extracts on dendrites after Aβ(25-35)-induced atrophy. Cortical neurons were cultured for 3 days and then treated with 10 μM Aβ(25-35) or water (Cont). Three days after the administration of Aβ(25-35), cells were treated with the methanol (A) or water (B) extract of *E. senticosus* (10–1000 ng/ml) or the vehicle (0.1% DMSO, Veh). Five days after treatment, the cells were fixed and immunostained for MAP2. The lengths of MAP2-positive neurites were measured. Values are means ± S.E.M. of data from 4 areas. *P<0.05, when compared with Veh.
Fig. 3. Effects of _E. senticosus_ extracts on synaptic reconstruction after Aβ(25-35)-induced synaptic loss. After cultivation for 21 days, the cortical neurons were treated with 10 μM Aβ(25-35) or water (Cont). Four days after the administration of Aβ(25-35), the cells were treated with the methanol (A, 100, 1000 ng/ml) or water (B, 10 – 1000 ng/ml) extract of _E. senticosus_ or vehicle (0.1% DMSO, Veh). Seven days after the drug treatment, the cells were fixed and double-immunostained for synaptophysin and MAP2. Areas of synaptophysin-positive puncta per 1 μm of dendrites were measured. Quantified data are shown. Values are means ± S.E.M. of data from 19 – 38 dendrites. *P<0.05, when compared with Veh.
and water extracts were able to regenerate axons and dendrites in Aβ(25-35)-treated neurons (Figs. 1 and Fig. 2), we next examined their effects on synaptic maturation. Rat cortical neurons were cultured for 21 days to allow development of mature synapses in vitro as described previously (8, 10). The cultures were treated for 4 days with Aβ(25-35). After that, cells were treated with extracts for 7 days. Dendritic shafts were visualized by double-immunostaining with antibodies to MAP2. Synaptophysin-positive puncta were present at the edge of the dendritic shafts. Quantification of the area synaptophysin-positive puncta on dendritic shafts showed that Aβ(25-35) caused a significant decrease in synapses (Fig. 3). These results indicate that Aβ(25-35) caused the loss of synaptic structures in long term-cultured cortical neurons. This was increased by treatment with the methanol extract (100, 1000 ng/ml; Fig. 3A) and the water extract (10, 100 ng/ml; Fig. 3B). NGF had no effect on the presynaptic density (Fig. 3C). No distinct difference of potencies of the methanol extract and water extract for increasing presynaptic density was observed.

**Protection from neuronal death**

Aβ(25-35) is known to induce neuronal cell death, especially in vitro (14). We therefore investigated the effects of the extracts on cell survival (Fig. 4). Treatment of cortical neurons with 10 μM Aβ(25-35) for 3 days caused substantial cell death. Addition of the methanol and water extracts (10 – 1000 ng/ml) along with Aβ(25-35) for 5 days significantly protected the neurons from cell death.

**Fig. 4.** Effects of *E. senticosus* extracts on cell survival in Aβ(25-35)-induced damage. After cultivation for 3 days, the cortical neurons were treated with 10 μM Aβ(25-35) or water (Cont). At the same time, the cells were treated by the methanol (A, 10 – 1000 ng/ml) or water (B, 10 – 1000 ng/ml) extract of *E. senticosus*, NGF (C, 100 ng/ml), or vehicle (0.1% DMSO, Veh). Five days after the extract treatment, cell viability was measured. Countable (attached) cell numbers in (A) and (B) were 233 (A, Cont), 172 (A, Veh), 217 (A, 10 ng/ml extract), 289 (A, 100 ng/ml extract), 279 (A, 1000 ng/ml extract), 233 (B, Cont), 172 (B, Veh), 315 (B, 10 ng/ml extract), 275 (B, 100 ng/ml extract), 258 (B, 1000 ng/ml extract). Values are means ± S.E.M. of data from 4 photographs. *P<0.05, when compared with Veh.
Contents of three major constituents in the methanol and water extracts

We measured the contents of three major constituents, eleutherosides B and E and isoflaxidin, in the methanol and water extracts by the HPLC method. Contents of these compounds were more in the methanol extract than the water extract (Table 1).

Eleutheroside B protects against atrophies of axons and dendrites

We examined effects of eleutherosides B and E and isoflaxidin on neurite atrophy induced by Aβ(25-35). The compounds were administered with Aβ(25-35), and axon and dendrite lengths were measured after 5 days. To detect activities more clearly compared with post-application, compounds were simultaneously administered with Aβ(25-35). We found that the axon (Fig. 5: A and C) and dendrite (Fig. 5: B and D) lengths were shorter in the cells treated with Aβ(25-35) followed by vehicle than in control cells. Lengths of axons and dendrites were significantly enhanced by co-applied eleutheroside B in a dose-dependent manner (1, 10 μM). Although eleutheroside E and isofraxidin also induced slighter extensions of axons and dendrites, those effects were less than that of NGF (100 ng/ml), a positive control.

Discussion

In the brain of Alzheimer’s disease, it is well known that cholinergic neurons are selectively lost (19). Based on this fact, cholinesterase inhibitors such as donepezil were developed as anti-Alzheimer’s disease drugs and are clinically used at the present. However, these cholinesterase inhibitors just slow the progress of the disease, but do not cure it (20, 21). This may be because supplying acetylcholine is not sufficient under conditions where there has been severe progression of neuritic atrophy, synaptic loss, and neuronal death. This study showed for the first time the effects of extracts of *E. senticosus* on neuronal morphological plasticity and cell damage under the Aβ(25-35)-induced neurodegenerative condition using rat cortical neurons. In Alzheimer’s disease, neuritic atrophy and synaptic loss occur prior to the neuronal death event and may be the first trigger of the memory impairment (22). In our preliminary experiments, the methanol extract, which was administered per orally, improved spatial memory deficit in Aβ(25-35)-injected mice.

Although the extracts of *E. senticosus* inhibited Aβ(25-35)-induced neuronal death, this may not be the sole reason that the extract promoted neurite extension and presynaptic density because extracts were administered to cells after treatment with Aβ(25-35) when the
Fig. 5. Protective effects of eleutheroside B, eleutheroside E, and isofraxidin on Aβ(25-35)-induced atrophies of axons and dendrites. Cortical neurons were cultured for 3 days and were then treated with 10 μM Aβ(25-35) or water (Cont). At the same time, the cells were treated by eleutheroside B, eleutheroside E, isofraxidin (1 and 10 μM), NGF (100 ng/ml), or vehicle (0.1% DMSO, Veh). Five days after treatment, the cells were fixed and immunostained for phosphorylated NF-H (A, C) or MAP2 (B, D). The lengths of phosphorylated NF-H-positive or MAP2-positive neurites were measured. Representative photographs are shown with a scale bar. Values are means ± S.E.M. of data from 5 – 9 photographs. *P<0.05, when compared with Veh.
damage had already occurred (8). Aβ(25-35) evoked neuritic abnormality and cell death in our experiments at the cellular level. These two phenomena seem to be mediated by different cellular signaling pathways. Heredia et al. reported that Aβ(1-40) or Aβ(25-35) induced dramatic reduction in the axonal network and the dystrophy related to actin remodeling in the aberrant focal adhesion complex mediated by activations of LIM kinase and coflin (23). Aβ(1-42)-induced axonal degeneration was also inhibited by a calpain inhibitor in an apoptosis-independent manner (24). In contrast, cell death triggered by Aβ seems to be mediated by other cellular mechanisms. Aβ(1-40) and Aβ(25-35) evoke cultured cortical and hippocampal cell death associated with caspase-3 activation (25). Caspase inhibitors block Aβ(1-42)-induced apoptosis (24). Aβ(25-35)-induced cell death is also known to be mediated by c-Jun N-terminal kinase activation (26). In our experiments (16), Aβ injection into the brain elicits no apparent neuronal death, although treatment with Aβ induces cell death in culture. However, the neuroprotective effect of E. senticosus must be advantageous in the patient’s brain where neuronal death is severely progressing.

Density of presynapses was increased by the methanol and water extracts of E. senticosus. For example, the activation of tyrosine kinase receptor B (27, 28) and NMDA receptor-mediated retrograde transport of nitric oxide (29) were proposed as mechanisms of presynaptic formation. Neurite outgrowth effect by NGF has been shown in many reports (30, 31), and activations of at least Rac1 (32) and Ras (33) were suggested as molecules related to the neurite outgrowth. However, several reports indicated NGF could not enhance expressions of molecules related to the neurite outgrowth. However, several reports indicated NGF could not enhance expressions of synaptophysin and synapsin I in Xenopus nerves (34) and synaptic currents in rat visual cortical slices (35).

Although we are not able to specify the molecular mechanism of extracts of E. senticosus at present, a variety of constituents, but not only one, may activate those multiple signal cascades for neuritogenesis, synaptogenesis, and cell survival.

Potencies of the methanol extract and water extract against inhibiting neurite atrophy, synaptic loss, and cell death were not clearly different. Although eleutherosides B and E and isofraxidin were measured for standardizing extracts, their contents were less in the water extract compared with the methanol extract. Eleutheroside B showed good protective effects on axonal and dendritic atrophies. At 10 μM, eleutheroside B–treated cells extended axons and dendrites of longer length than those in the control cells. Although eleutheroside B and isofraxidin weakly protected against those atrophies at 10 μM, the content of eleutheroside B and isofraxidin can be calculated to be approximately 0.048 and 0.01 μM, respectively, even in the methanol extract (1000 ng/ml).

Although it was reported that eleutheroside E and isofraxidin suppressed proinflammatory mediators in synovial sarcoma cells (36), activities of eleutheroside B and other compounds from E. senticosus in the nervous system have not been investigated. Although eleutheroside B is suggested to be one of active compounds in E. senticosus, similar potencies of the methanol and water extracts for neuronal damages are not yet explained by eleutheroside B. We have also a possibility of finding some other active constituents in E. senticosus.

In conclusion, we found that E. senticosus extracts regenerated axons, dendrites, and synapses and attenuated neuronal damage in the culture model of Alzheimer’s disease–like neurodegeneration. E. senticosus is widely used as easily available adaptogenic supplements. However, E. senticosus may be a candidate useful for developing therapeutic drugs for Alzheimer’s disease.

Acknowledgments

This work was partially supported by Grants-in-Aid for Scientific Research (B) (17406004) from the Japan Society for the Promotion of Science as well as by the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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