Characterization of Anti-neurodegenerative Effects of Polygala tenuifolia in Aβ(25–35)-Treated Cortical Neurons

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Received April 14, 2006; accepted June 2, 2006; published online June 9, 2006

Although Polygala tenuifolia Willd (PT) was classically mentioned as an anti-dementia drug in Chinese and Japanese traditional medicine, basic research showed only enhancement of the cholinergic function. In Alzheimer’s disease, neuritic atrophy and synaptic loss occur prior to neuronal death event, and may be the first trigger of the memory impairment. Therefore, we studied effects of Polygala tenuifolia Willd (PT) on Aβ(25–35)-induced neuronal damage using rat cortical neurons for characterization of activities of PT under Aβ-induced neuronal damage. Treatment with the water extract of PT enhanced axonal length dose-dependently after Aβ(25–35)-induced axonal atrophy. However, dendritic atrophy and synaptic loss induced by Aβ(25–35) were not recovered by treatment with PT extract. In contrast, Aβ(25–35)-induced cell damage was completely inhibited by PT extract. By characterization of PT effects on neuronal morphological plasticity and cell damage, usefulness as well as an insufficiency of PT as an anti-dementia drug was clarified.

Key words Polygala tenuifolia; axon; dendrite; synapse; neuronal damage; Alzheimer’s disease

The root of Polygala tenuifolia Willd (PT) (Japanese name: Onji) is a well-known traditional Chinese medicine used as an expectorant, a tonic, a tranquilizer and an anti-dementia drug. However, basic researches of the anti-dementia effects are very few in spite of PT being generally recognized as a good herbal drug for amnesia. It was reported that 80% ethanol extract of PT and the methanol extract of PT reverse cognitive impairments in rats.1,2) Acetylcholinesterase (AChE) activity is inhibited in vitro by 80% ethanol extract of PT.1,3) Onjisaponin F, a constituent in PT, increases cholineacetyltransferase (ChAT) mRNA level in rat basal forebrain cell, and. Taken together, transient enhancement of cholinergic systems by PT may be involved in the memory improvement seen in like the scopolamine model. However, practical dementia is caused by irreversible neuronal damage involving neuronal death, neuritic atrophy and synaptic loss. Especially in case of Alzheimer’s disease, neuritic atrophy and synapse loss are earlier events than neuronal death are, and are critical for memory disorder.4–8) Previous our studies suggested that compounds which showed synaptic regeneration activity in cultured neurons were also active for memory impairment in vivo using Alzheimer’s disease model mice.9–11) Although protective effects of PT on neuronal death were shown in amyloid β (Aβ), glutamate or NMDA-induced neuronal damage, effects of PT on atrophy of neuritis and synaptic loss never have been investigated yet.1,12) Therefore, we aimed in this study to characterize activities of PT under Aβ-induced neuritic damage.

MATERIALS AND METHODS

Materials Thirty gram of roots of Polygala tenuifolia Willd (PT) was extracted with 600 ml of water at 100 °C for 40 min. This crude drug was purchased from Tochimoto Tenkaido (Osaka, Japan). The decoction was evaporated under reduced pressure and freeze-dried of extract powder (yield: 8.02 g). The extract was then dissolved in sterilized distilled water at 1000 times concentrations of final concentrations. Aβ(25–35) (Sigma, Saint Louis, MO, U.S.A.) was dissolved in sterile distilled water at a concentration of 5 mM, and was incubated at 37 °C for 3 d to allow fibril formation. [Gly35]-Humanin was purchased from Phoenix Pharmaceuticals (Belmont, CA, U.S.A.) as a positive control for neuronal protection. A monoclonal antibody to phosphorylated neurofilament-H (NF-H) was purchased from Sternberger Monoclonals (Lutherville, MD, U.S.A.). A monoclonal antibody to microtubule-associated protein 2 (MAP2), an antisem to MAP2, polyclonal antibody to MAP2 and a monoclonal antibody to synaptophysin were purchased from Chemicon (Temecula, CA, U.S.A.). Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 546-conjugated goat anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR, U.S.A.). Aqua Poly Mont was purchased from Polyscience (Warrington, PA, U.S.A.).

Primary Culture Embryos were removed from pregnant Sprague Dawley rats (Japan SLC, Shizuoka, Japan) at 17 or 19 d of gestation. The animals were handled in accordance with the Guideline for the Care and Use of Laboratory Animals of University of Toyama. The cortices were dissected, and the dura mater was removed. The cells minced and dissociated were plated on 8-well chamber slides or 12-well plate (Falcon, Franklin Lakes, NJ, U.S.A.) coated with poly-D-lysine (5 μg/ml) at 37 °C in a humidified incubation with 10% CO2 with Neurobasal media including 12% horse serum. The density of the cells was 1.0×105 cells/ml for short term culture, or 1.5×106 cells/ml for long term culture.

Drug Treatment The cells were cultured in medium containing serum for short-term culture or in medium containing 2% B-27 supplement without serum for long-term culture. In case of short-term culture, the cells were cultured for 3 d and were treated with 10 μM Aβ(25–35) for 4 d. After that, cells were treated extract of PT extract at each concentration for 5 d. In case of long-term culture, half of the medium in each well was replaced with serum-free medium containing the 2% B-27 supplement at 3 d after initiation of the culture period. At every 7 d of culture, half of the medium was replaced with fresh serum-free medium. The
cells were cultured for 21 d and were treated with 10 μM Aβ(25–35) for 4 d. After that, cells were further treated with PT extract for 7 d. The detailed time schedules of the experiments are shown below the figures.

**Analysis of Neurite Outgrowth** Rat cortical neurons on 8-well chamber slides were fixed by 4% paraformaldehyde and immunostained with a monoclonal antibody to phosphorylated neurofilament-H (NF-H) (dilution 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, Japan) at 425 nm. The length of neurites positive for phosphorylated NF-H or MAP2 was measured using an image analysis software Neurocyte (Kurabo, Osaka, Japan).

**Analysis of Synaptic Formation** After the drug treatment, the cells were fixed and double-immunostained with a combination of monoclonal antibody to synaptophysin (dilution 1:500) as a presynaptic marker and a polyclonal antibody to MAP2 (dilution 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, U.S.A.) 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, Japan). The length of the dendrites was measured using Image J software (http://rsb.info.nih.gov/ij/index.html).

**Cell Viability Assessment** Cell viability was determined by calcein staining. Cells on 8-well chamber slides were rinsed by phosphate-buffer saline (PBS), and were incubated with 6 μM calcein AM (Dojindo, Kumamoto, Japan) for 40 min at 37 °C. After rinsing by PBS, cells were fixed by 4% paraformaldehyde and mounted. Fluorescence images and bright field images were simultaneously captured (four images per treatment) by AX-80 microscope. The percentage of dead cell was valued as the ratio of dead cells (calcein-positive) to total cells. The total cell number was counted in bright-field photos.

**Statistical Analysis** Statistical comparisons were performed by the Student’s t-test, or one-way analysis of variance followed by Bonferroni’s method. Values of p<0.05 were considered significant. The means of the data presented together with S.E.M.

**RESULTS**

**Extract of P. tenuifolia Activates Extension of Axons, But Not Dendrites, after Aβ(25–35)-Induced Damage** Rat cortical neurons were cultured with Aβ(25–35) for 4 d, then PT extract (0.1, 1, 10, 100 μg/ml) or vehicle (water) was added. After drug treatment for 5 d, cells were fixed and immunostained for phosphorylated NF-H or MAP2. Both axons (Fig. 1) and dendrites (Fig. 2) were significantly shortened after treatment with Aβ(25–35). Treatment with PT extract resulted in a significant increase in axon in a dose-dependently manner (Fig. 1). One way ANOVA revealed a significant dose-dependent effect in the extract-treated group (p=0.00000747). The length of axons reached to the same level of control by treatment with 1 μg/ml PT extract. Higher doses were more effective. In contrast, Aβ(25–35)-induced atrophy of dendrites was not recovered by treatments with PT extract at any doses (Fig. 2).

**Extract of P. tenuifolia Had No Effect on Reconstructing Synapses in Damaged Neurons** Rat cortical neurons were cultured for 21 d to construct mature synapses, and then Aβ(25–35) was added to the cells to induce synaptic loss. Four days later, PT extract (100 μg/ml), nerve growth factor (NGF; 100 ng/ml), or vehicle (water) was added to the cells. Seven days later, the cells were fixed and double-immunostained for synaptophysin and MAP2. Synaptophysin-positive areas in vehicle-treated neurons at 11 d after treatment with Aβ(25–35) (7.6±0.22 μm²/μm dendrite) were significantly reduced compared with control (11.8±0.23 μm²/μm dendrite) (Fig. 3). Treatment with PT extract did not increase synaptophysin-positive areas (7.2±0.17 μm²/μm dendrite), indicating that PT extract had no synaptogenesis activity. NGF treatment also did not increase synaptophysin-positive areas (6.67±0.42 μm²/μm dendrite), which is consistent with previous results.10,11,13

**Extract of P. tenuifolia Attenuates Aβ(25–35)-Induced Cell Damage** Protective effects of PT extract on Aβ(25–35)-induced cell damage were investigated. Rat cortical neurons were treated with Aβ(25–35). After 4 d, PT extract or vehicle (water) was treated. Five days after the extract treatment, cell viability was determined by measuring calcein uptake. [Gly14]-Humanin peptide was shown to be effective on Aβ(25–35)-induced cell damage at lower dose (10 nM) compared with native form of the peptide.12 Rate of damaged cells was increased at 9 d after Aβ(25–35) treatment compared with 4 d after (Fig. 4B). At a dose of 100 μg/ml, PT extract suppressed the Aβ(25–35)-induced cell damage to the level before extract treatment. Treatments with lower dose of PT extract (10 μg/ml) and [Gly14]-Humanin (10 nm) inhibited completely the Aβ(25–35)-induced cell damage (Fig. 4B). Detached cells from the dish bottom indicated irreversible cell death after severe damage. In Fig. 4C, attached cell numbers slightly, but not significantly decreased in Aβ(25–35)-treated cells. PT extract had no protective effect on the cell death. In contrast, treatment with [Gly14]-Humanin almost completely inhibited the severe cell death.

**DISCUSSION**

In the brain of Alzheimer’s disease, it is well known that cholinergic neurons are selectively lost.15 Based on the 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 play a role just slowing the progress of disease, but not curing it.16,17 It may be due to that supplying acetylcholine is not expected when neuritic atrophy, synaptic loss and neuronal death are progressed severely. Although PT was classically...
Fig. 1. The Effect of PT Extract on Axons after Aβ(25—35)-Induced Atrophy

Cortical neurons were cultured for 3 d and were then treated with or without (Cont) 10 μM Aβ(25—35). Four days after the administration of Aβ(25—35), cells were treated by PT extract (0.1—100 μg/ml) or vehicle (0.1% DMSO, Veh). Five days after treatment, the cells were fixed and immunostained for phosphorylated NF-H. The lengths of NF-H-positive neurites were measured. Photographs of stained cells (A) and quantified data (B) are shown. Values are means±S.E.M. of data from 10—15 cells. *p<0.05 when compared with Veh. Scale=100 μm.

Fig. 2. The Effect of PT Extract on Dendrites after Aβ(25—35)-Induced Atrophy

Cortical neurons were cultured for 3 d and were then treated with or without (Cont) 10 μM Aβ(25—35). Four days after the administration of Aβ(25—35), cells were treated by PT extract (0.1—100 μg/ml) or 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. Photographs of stained cells (A) and quantified data (B) are shown. Values are means±S.E.M. of data from 12—18 cells. *p<0.05 when compared with Veh. Scale=100 μm.

Fig. 3. The Effect of PT Extract on Synaptic Reconstruction after Aβ(25—35)-Induced Synaptic Loss

After cultivation for 21 d, the cortical neurons were treated with or without (Cont) 10 μM Aβ(25—35). Four days after the administration of Aβ(25—35), the cells were treated with PT extract (100 μg/ml) or vehicle (0.1% DMSO, Veh). Seven days after the drug treatment, the cells were fixed and double-immunostained for synaptophysin (green) and MAP2 (red). Areas of synaptophysin-positive puncta per 1 μm of dendrites were measured. Photographs of stained cells (A) and quantified data (B) are shown. Values are means±S.E.M. of data from 75—115 dendrites. *p<0.05 when compared with Veh. Scale=50 μm.
PT extract strongly inhibited Aβ(25—35)-induced cell damage. The inhibitory effect of PT extract was shown when added 4 d after the treatment with Aβ(25—35) (Fig. 4). In Fig. 4B, cell viability was measured in control cells, and was recovered to the same level of control by 10 µg/ml PT extract in spite of post treatment, suggesting that the cell damage is reversible, and the 10 µg/ml PT extract may have stronger effect on repair the cell damage than 100 µg/ml PT extract. [Gly14]-Humanin peptide also had repair effect on Aβ(25—35)-induced cell damage. Somehow, 10 µg/ml PT extract was more effective than 100 µg/ml extract in neuronal protection. In contrast, axonal extension was the most effective by treatment with 100 µg/ml PT extract. In addition, dendritic atrophy and synaptic loss were not improved by any doses of PT extract. These results suggest that the axonal

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growth effect by PT extract is not caused just as a result of increase in survived cells, and mechanisms of PT extract of axonal growth, dendritic growth, synaptic formation and cell protection may be independent each other. Previous other reports suggest that onjiasaponin-F increased ChAT mRNA level, and tenuifoliside B improved scopolamine-induced memory deficit. Identification of active constituents for axonal regeneration and neuronal protection will be carried out future.

$\text{A}\beta (25-35)$ is not found in the Alzheimer’s disease brain. However, several reports that $\text{A}\beta (25-35)$ is an active partial fragment of $\text{A}\beta$. This fragment also forms a $\beta$-sheet structure and induces neuronal death and $\text{A}\beta$ neurotrophic.1,21) synaptic loss.1,18,21) Also in our previous paper, it was confirmed that effects of $\text{A}\beta (25-35)$ and $\text{A}\beta (1-42)$ on neuritic atrophy and cell death were seen similarly. 10 $\mu M$ $\text{A}\beta (1-42)$ induced axonal and dendritic atrophy as well as 10 $\mu M$ $\text{A}\beta (25-35)$ did. The degree of the atrophy induced by $\text{A}\beta (25-35)$ wasn’t different from $\text{A}\beta (1-42).$ 10) Although it was reported that mechanisms of oxidative stress might be different in $\text{A}\beta (1-42)$ and $\text{A}\beta (25-35),$ 22) we believe that varieties and aspects of resultant damages by those two peptides are quite similar. However, effects of PT extract should be investigated in future using $\text{A}\beta (1-42)$-induced neurodegeneration models $\text{in vitro}$ and $\text{in vivo}$.

The root of PT is practically used as one of crude drugs of Kampo formulation in Japan. The greatest merit of Kampo formulation is harmonized and synergic effects brought by each crude drug combined. Since PT has activities of axonal regeneration and neuronal protection in addition to cholinergic enhancement, other crude drugs which have effects on dendritic regeneration and synaptic formation, may comple- lieve that varieties and aspects of resultant damages by those two peptides are quite similar. However, effects of PT extract should be investigated in future using $\text{A}\beta (1-42)$-induced neurodegeneration models $\text{in vitro}$ and $\text{in vivo}$.

REFERENCES