Extract prepared from the hooks and stems of *Uncaria sinensis* prevents glutamate-induced neuronal death in cultured cerebellar granule cells

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Abstract

Uncaria sinensis (OLIV.) HAVIL. (US), Uncariae Uncus Cum Ramulus, is a medicinal plant used in Japan for the treatment of various symptoms accompanying hypertension and cerebrovascular disorders. We studied the protective effect of its hooks and stems on glutamate-induced neuronal death by microscopic observation and MTT assay, and action on $^{45}Ca^{2+}$ influx using cultured cerebellar granule cells from 7-8 day-old rats. Glutamate-induced cell death was protected by the application of water extract of *Uncaria sinensis* (USE) in a dose-dependent manner, and concentrations of 10^{-5} to 10^{-4} g/ml had a significant effect compared to exposure of glutamate only. Further, the increase of $^{45}Ca^{2+}$ influx into cells by glutamate was also blocked by USE in a dose-dependent manner, and concentrations of 10^{-5} to 10^{-4} g/ml were significant. These results suggest that US has a protective effect on glutamate-induced neuronal death in cultured cerebellar granule cells through the inhibition of Ca^{2+} influx.

Key words *Uncaria sinensis* (OLIV.) HAVIL., Uncariae Uncus Cum Ramulus, glutamate, calcium, cultured cerebellar granule cells.

Abbreviations US, *Uncaria sinensis* ; USE, water extract from *Uncaria sinensis* ; NMDA, *N*-methyl-D-aspartate ; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide ; AP5, 2-amino-5-phosphonovaleric acid.

Introduction

Glutamate is a physiological excitatory amino acid transmitter in the central nervous system. However, overactivation of glutamate receptors has been suggested to be involved in several neurological disorders including ischemic-hypoxic injury,[1] epilepsy,[2] and neurodegenerative disease.[3] The extracellular concentration of glutamate elevates in the brain during ischemia.[4] Excessive release of glutamate resulting from ischemia overstimulates glutamate receptors and induces neuronal death through Ca^{2+} influx.[5] Disruption of this homeostasis can lead to cerebrovascular disorders and dementia. Glutamate receptors can be classified as metabotropic and ionotropic receptors.[6] Overactivation of metabotropic glutamate receptors has not been found to be toxic in neuronal culture.[7] On the other hand, overstimulation of ionotropic glutamate receptors, which include *N*-methyl-D-aspartate (NMDA) receptor and non-NMDA receptors, has been found to be toxic through Ca^{2+} influx in neuronal culture.[5] Cultured cerebellar granule cells also have glutamate receptors.[8] Excessive activation of glutamate receptor results in death in cultured cerebellar granule cells.[9] This glutamate - induced neuronal damage can be blocked by Mg^{2+} and NMDA receptor antago-

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nist such as 2-amino-5-phosphonovaleric acid (AP5).[10,12] Neuroprotective effects of drugs including a Kampo formulation, Toki-shakuyaku-san (当帰芍薬散), were evaluated using cultured cerebellar granule cells.[13-15]

We previously demonstrated by a well-controlled and double-blind study that a Japanese traditional (Kampo) medicine, called Choto-san (釣藤散), was effective in treating vascular dementia.[16,17] Choto-san is a Kampo formulation composed of 11 crude drugs.[18] The hooks and stems of *Uncaria sinensis* (OLIV.) HAVIL. (US) (Uncariae Uncus Cum Ramulus) is the main medicinal plant of Choto-san. US have been used in Japan for the treatment of many symptoms accompanied by hypertension and cerebrovascular disorders, proved to have hypotensive and vasodilative effects.[19-21] Further, oral administration of US extract proved to have anti-convulsive effects on glutamate-induced convulsion in mice.[22] This suggests that US may have some protective effects on glutamate-induced neuronal damage. Therefore, we used an experimental system with cultured cerebellar granule cells to confirm this possibility.

Materials and Methods

Cell culture : Cerebellar granule cells were cultured as described by Gallo *et al.*[23] In brief, about ten cerebella were dissected from the brains of 7-8 day-old Wistar rats, chopped into small blocks and placed into 25 ml of Krebs Ringer buffer solution (KRB). This was centrifuged at 150 g for 30 sec, the pellet was resuspended in 25 ml of KRB containing 0.025 % trypsin (Sigma, St. Louis, MO, USA), and incubated at 37°C for 13 min. The trypsinization was stopped by addition of 0.005 % trypsin inhibitor (Sigma) with 0.01 % deoxyribonuclease (Sigma). The resulting pellet was centrifuged and dissociated into cells, and suspended in basal Eagle medium (Sigma) containing 10 % fetal bovine serum (Sigma), 2 mM glutamine, 20 μg/ml of gentamicine (Sigma) and 25 mM KCl. The cells were seeded at a density of 1.25×10^6 cells/ml in 35 mm poly-L-lysine coated culture dishes (Iwaki, Tokyo, Japan). The cultures were maintained at 37°C with 5 % CO_2 in a humidified incubator. Cytosine arabinoside (Sigma) (10 μM) was added 18 h after plating to prevent proliferation of glial cells. The culture medium was not changed thereafter. The cultured cells were used at 7-8 days *in vitro* (DIV) for the experiments.

Preparation of extract from Uncaria sinensis : The water extract from *Uncaria sinensis* (OLIV.) HAVIL. (USE) was prepared from the hooks and stems of US purchased commercially (Guangxi, China origin, Tochimoto Pharmaceuticals, Osaka, Japan). US (100 g) was boiled in water (500 ml) for 50 min. This solution was centrifuged at 10,000 g for 30 min, and the supernatant was then converted to freeze-dried powder (7.7 g). This extract was dissolved in the corresponding solution and filtered using a 0.20 μm filter unit (Iwaki) before application to cells.

Cell viability : Cell viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) staining essentially as described previously.[24] Cultured cells were washed 3 times with Mg^{2+}-free Locke's solution (in mM : 154 NaCl, 5.6 KCl, 3.6 $NaHCO_3$, 5.0 HEPES, 2.3 $CaCl_2$, 5.6 glucose, pH 7.4), then incubated with Mg^{2+}-free Locke's solution with (control) or without (vehicle) 100 μM L-glutamic acid (glutamate) (Sigma). AP5 (RBI, Natick, MA, USA) (100 μM), a specific NMDA receptor antagonist, and various concentrations of USE (10^{-6}-10^{-4} g/ml) were dissolved in Mg^{2+}-free Locke's solution together with glutamate (100 μM), and then applied to cells. The concentrations of glutamate and AP5 applied to the cells were determined according to the previous studies.[12,15] After 1 h incubation at 37°C, MTT (Sigma) (500 μg/ml) was applied and incubated for 30 min at 37°C. Cells were then washed and lysed in isopropanol with 0.04 N HCl to dissolve the blue formazan products. Optical density was read at 570 nm with a spectrophotometer and expressed as percentage of the vehicle.

It is known that Mg^{2+} blocks the activation of glutamate to NMDA receptor.[10,11] For the purpose of evaluating the effect of Mg^{2+} containing USE against glutamate-induced neuronal death, we checked the Mg^{2+} concentration of USE solution and examined the effect of Mg^{2+} on cell viability.

$^{45}Ca^{2+}$ influx : Influx of $^{45}Ca^{2+}$ was measured as previously described.[25] Granule cells were incubated

for 1 h in Mg^{2+}-free Locke's solution containing drugs and $^{45}CaCl_2$ (Amersham, Little Chalfont, Buckinghamshire, UK) (1 μCi/ml) at 37°C, and washed 3 times with Ca^{2+}- and glucose-free Locke's solution containing 2 mM EGTA. The incubation solution was discarded and cells were solubilized with 1 N NaOH. The amount of radioactivity incorporated by the cells was measured by liquid scintillation counter and expressed as fold-increase vs. vehicle.

Statistical analysis : Three or more dishes were used in each group and their mean was calculated in each experiment. Values are mean±S.D. from four separate experiments. The data were compared by Student's t-test. A level of $p<0.05$ was accepted as statistically significant.

Results

Cultured cerebellar granule neurons at 7-8 DIV were observed as clear round cells surrounded by a network of neurites by phase contrast microscopy (Fig. 1a). Glutamate exposure (100 μM, 1 h) -induced neuronal degeneration appeared as swelling of the soma, punctiform granulations in the soma and bright pycnotic mass of nucleus (Fig. 1b). These signs of neuronal death were prevented by AP5 (100 μM), a specific NMDA receptor antagonist (Fig. 1c). USE (10^{-5}-10^{-6} g/ml) also protected cells from this glutamate-induced neuronal damage (Fig. 1d).

Cell viability was evaluated by MTT assay (Fig. 2). MTT is converted to an insoluble blue formazan product by various dehydrogenases within mitochondria in living cells but not in dying cells.[24] Glutamate (100 μM, 1 h) -induced neuronal death was fully prevented by AP5 (100 μM). The incubation of granule cells together with glutamate and USE was found to be protective for the cells in a dose-dependent manner, and concentrations of 10^{-5} to 10^{-4} g/ml of USE showed significant protection compared to only glutamate exposure.

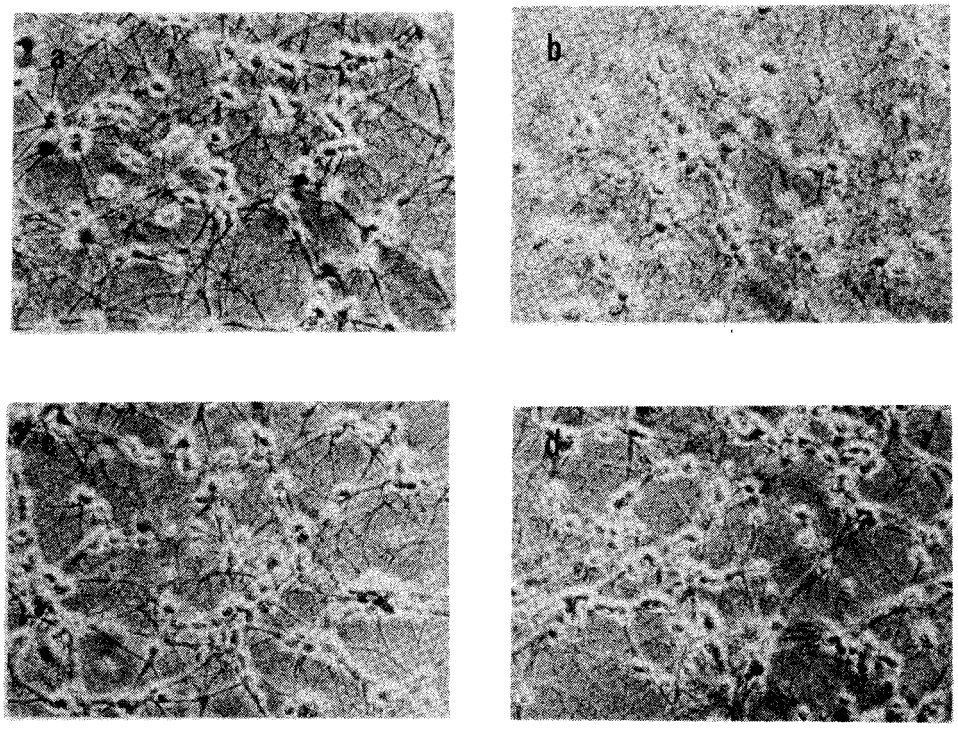

Fig. 1 Phase contrast photographs of cerebellar granule cells at 8 DIV 1 h after application of various drugs. a, Vehicle (Mg^{2+}-free Locke's solution). b, Glutamate (100 μM). c, AP5 (100 μM) with glutamate (100 μM). d, Extract of *Uncaria sinensis* (3×10^{-5} g/ml) with glutamate (100 μM).

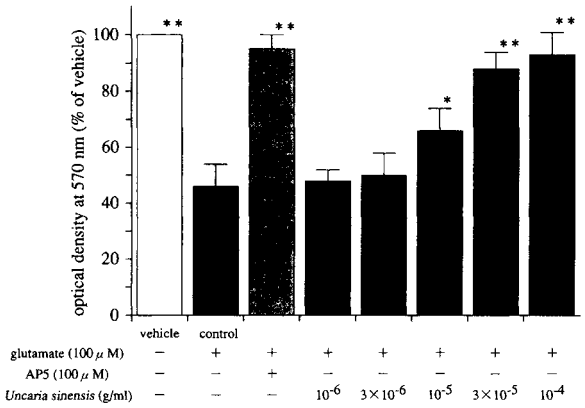

Fig. 2 Effect of extract of *Uncaria sinensis* on cell viability against glutamate-induced neuronal damage assessed by MTT assay in cerebellar granule cells. Cells were incubated in various drugs for 1 h. Three or more dishes were used in each group and their mean was calculated in each experiment. Values are mean±S.D. from four separate experiments. $*p<0.05$, $**p<0.01$ (compared to control).

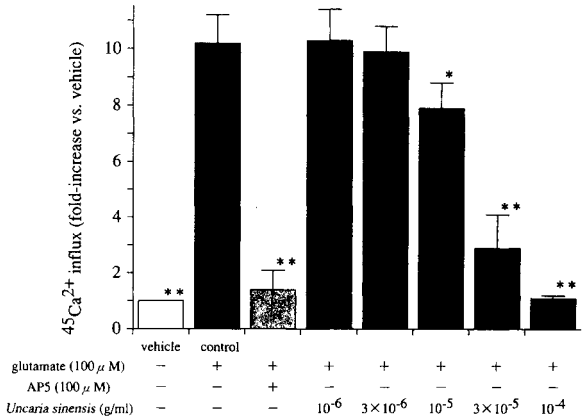

Fig. 3 Effect of extract of *Uncaria sinensis* on glutamate-induced $^{45}Ca^{2+}$ influx into cerebellar granule cells. Cells were incubated in various drugs for 1 h. Three or more dishes were used in each group and their mean was calculated in each experiment. Values are mean±S.D. from four separate experiments. $*p<0.05$, $**p<0.01$ (compared to control).

Using $^{45}Ca^{2+}$ as a radioactive tracer, we next quantified the Ca^{2+} influx under various conditions. As shown in Fig. 3, glutamate (100 μM) exposure (1 h) increased $^{45}Ca^{2+}$ influx about ten-fold vs. vehicle, and AP5 (100 μM) prevented glutamate-induced $^{45}Ca^{2+}$ influx. USE also prevented $^{45}Ca^{2+}$ influx induced by glutamate in a dose-dependent manner, and concentrations of 10^{-5} to 10^{-4} g/ml were significant compared to only glutamate exposure.

The Mg^{2+} concentration of the USE solution (10^{-4} g/ml) used in this study, which was the maximum effective dose against glutamate-induced neurotoxity, was 1.89×10^{-2} mM. We added 1.89×10^{-2} mM Mg^{2+} to Mg^{2+}-free Locke's solution, and examined the protective effect of this solution on glutamate-induced cell death, but there was no apparent effect (data not shown).

Discussion

The present study clearly demonstrated that USE prevents glutamate-induced neuronal death in cultured cerebellar granule cells through the inhibition of Ca^{2+} influx.

Glutamate is the major excitatory neurotransmitter in the central nervous system, and plays an important role in learning and memory. However, brain ischemia leads to excessive release of glutamate and neuronal death through Ca^{2+} influx.[4,5] Neuronal cells have different types of ionotropic glutamate receptors, NMDA receptor and non-NMDA receptors, which include α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor and kainate receptor.[26] NMDA receptor is characterized by high Ca^{2+} permeability, and the other two receptors have very low Ca^{2+} permeability but still are concerned with Ca^{2+} permeability through membrane depolarization. Further, neuronal cells have metabotropic glutamate receptors, voltage-dependent Ca^{2+} channels, and Ca^{2+} storages.[27,28] Overactivation of metabotropic receptors has not been found to be toxic.[7] On the other hand, overstimulation of ionotropic glutamate receptors has been found to be toxic through Ca^{2+} influx.[5] AP5, which was used as positive control in this study, is a specific NMDA receptor antagonist and has a protective effect against glutamate-induced neuronal death by blocking Ca^{2+} influx through ion channel of NMDA receptor.[12]

It is known that Mg^{2+} blocks the action of glutamate to NMDA receptor.[10,11] US is a crude drug, so it probably contains Mg^{2+} to some extent, and the neuroprotective effect of USE seen in this study was possibly due to the Mg^{2+} contained in USE. In order to

confirm this, we checked the Mg^{2+} concentration of the USE solution. Further, we added Mg^{2+}, which corresponded to the Mg^{2+} concentration of USE solution, to Mg^{2+}-free Locke's solution, and examined the protective effect of this solution on glutamate-induced cell death, but there was no apparent effect. Accordingly, we conclude that the neuroprotective effect of USE is not due to the Mg^{2+} in USE, but to other components.

Mimaki *et al.*[22] reported that oral administration of Choto-san extract to mice tended to inhibit the glutamate-induced convulsion in a dose-dependent manner and the effect of USE at a 3.0 g/kg dose was significant, while both the extracts showed no activity against the picrotoxine-induced, strychinine-induced, and electroshock convulsions. These suggest that US has a protective effect against glutamate-induced brain damage *in vivo*, and may support the results of our present study that USE prevents glutamate-induced neuronal death in cultured neurons. Further, they also demonstrated that active components in US on glutamate-induced convulsion are such alkaloids as geissoschizine methylether and hirsuteine.[22] It was reported that the US has hypotensive and vasodilative effects,[19-21] and such alkaloids as hirsutine, rhynchophylline, isorhynchophylline, corynoxeine and isocorynoxeine in US exhibit Ca^{2+} channel blocking activity, perhaps mainly through the inhibition of the voltage-dependent Ca^{2+} influx, in isolated rat thoracic aorta.[29-31] The detailed mechanism about inhibitory effect of US on glutamate-induced Ca^{2+} influx in cultured neurons is not clear. Further studies need to focus on the search for the active components of US against glutamate-induced neuronal death and their action mechanisms at the molecular receptor levels.

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和文抄録

我々は，先に脳血管性痴呆に対する釣藤散の有用性を封筒法ならびに二重盲検臨床試験にて明らかにした。この作用機序の一端を解明する目的で，ラット培養小脳顆粒細胞を用いて，脳虚血による細胞死と関連するグルタミン酸誘導神経細胞死に対する，釣藤散の主要構成生薬である釣藤鈎の効果を検討した。7～8日齢のラットの小脳を摘出し，顆粒細胞を培養し7～8日目に実験を行った。MTT法による細胞生存率の実験では，釣藤鈎エキス（10^{-5}～10^{-4} g/ml）はグルタミン酸による細胞死を有意に抑制した。また，釣藤鈎エキス（10^{-5}～10^{-4} g/ml）は，グルタミン酸による $^{45}Ca^{2+}$ 流入を有意に阻害した。これらの効果は，いずれも濃度依存的であった。以上より，釣藤鈎はラット培養小脳顆粒細胞におけるグルタミン酸誘導神経細胞死を Ca^{2+} 流入阻害を介して抑制することが示唆された。

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