Neuroprotective Effect of Some Plant Extracts in Cultured CT105-Induced PC12 Cells

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Carboxyl-terminal fragments of APP (CT) have been found in plaques, microvessels and the neurofibrillary tangles in the brains of AD patients. These carboxyl-terminal fragments, which contain the complete Aβ sequence, appear to be toxic to neurons in culture cells. However, the possible role of other cleaved products of APP is less clear. We showed that a recombinant carboxy-terminal 105 amino acid fragment (CT105) of APP induced strong neurotoxicity in PC12 cells. We prepared alcoholic extract from Oriental herbal plants and screened their protective effects against CT_{105}-induced cell death in PC12 cells after the treatment of these extracts. Of the 10 kinds of plant extracts, 12 kinds of extracts had considerable protective effects against CT_{105}-induced cell death, especially, *Uncariae Ramulus et Uncus* (UREU), *Gastrodia elata* (GAE), *Evodia officinalis* (EO) and *Panax ginseng* (PAG) showed the most protective effect at the concentration of 50μg/ml. BuOH extract of UREU and GAE possessed the strongest protective effects against neurotoxicity of CT_{105}-induced PC12 cells and showed inhibitory effect with IC_{50} values of 4.8 and 8.3μg/ml, respectively. These plants are promising candidates of neuroprotective effects and would be useful for the treatment of the neuronal degenerative diseases such as Alzheimer's diseases.

Key words Alzheimer's disease; *Uncariae Ramulus et Uncus*; *Gastrodia elata*; beta-amyloid; neurodegeneration; CT105

A number of studies have reported that Aβ-bearing carboxyl-terminal fragments (CTs) have been found not only in senile plaques, paired helical filaments, and brain microvessels of AD patients but also in the white matter of Down's syndrome (DS) brains.[1)] Evidence for correlating CTs with neurodegeneration has come from cell transplantation models, transgenic mice, and the investigation of postmortem brains.[2—4)] Furthermore, a recombinant carboxyl-terminal fragment bearing the full-length Aβ (CT_{105}) elicited greater neurotoxic potency than Aβ on cultured neuronal cells and on memory deficiencies and neuropathological changes in mice.[5)]

Earlier studies showed that Aβ-bearing CT fragments were released from several different cells and/or more easily released from the damaged neurons into the medium or extracellular fluids.[6,7)] Moreover, a recent study reported that APP mutations found in familial AD increased the intracellular accumulation of potentially amyloidogenic and neurotoxic CTs in neurons.[8)] One potential mechanism underlying the neurotoxicity of these FAD mutants is that neuronal death occurs by deposition of Aβ or CT105. It has been reported by multiple research groups that intracellular signaling mechanisms, including oxidative stress-relevant pathways[9,10)] and caspase-dependent pathways,[11)] mediate Aβ amyloid-induced neurotoxicity.

Some herbal medicines had neuroprotective effect against Aβ or CT_{105}-induced cell toxicity, which might be beneficial for the therapeutic agent of Alzheimer's disease. Recently, several neuroprotective agents from plants were reported such as BT-11,[12)] EGb 761,[13)] Huperzine A[14)] and water extract of *Cinnamomum cassia.*[15)] The most of these compounds showed the protective effect of neurotoxicity through oxidative damage and cell death in neuronal cells.

In order to find new neuroprotective components from plant extracts, we tested several candidate extracts by MTT assay in CT_{105}-induced neuronal cells.

MATERIALS AND METHODS

Reagents and Materials Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin (FBS) were purchased from Gibco Laboratories (Detroit, MI, U.S.A.) and, isopropyl alcohol, 3-(4,5-diimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), poly-D-lysine (50 μg/ml in sterile water) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Plant Materials The plants were purchased from Kyengdong Herb market, Korea. Vouchers were deposited in the laboratory of natural research, Seoul National University.

Extraction and Solvent Fractionation Each plant was air-dried and ground. Then it was extracted three times with MeOH at room temperature and the solvent was evaporated under reduced pressure. The methanolic extracts of plant materials were dispersed in water and extracted with ethyl ether to get ether soluble fraction. Remained water layer was extracted again with EtOAc and *n*-BuOH, sequentially to yield EtOAc and BuOH soluble fractions.

Cell Culture PC12 cell line was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were grown in high-glucose DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, 1 μM pyruvate, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were grown at 37 °C, 5% CO_2 in fully humidified air, and were subcultured twice a week. The cells were grown on culture dishes precoated with poly-D-lysine (50 μg/ml in sterile water) overnight.

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CT-Induced PC12 Cell Toxicity The full-length cDNA for CT105 of human APP695 by RT-PCR was constructed into the BamH I/Hind III site in the pTRE, Tet-repressible expression plasmid. The Tet-on gene expression system was purchased from Clontech. (Palo Alto, CA, U.S.A.). After rat pheochromocytoma cell line, PC12 cells transfected with pTet-on vector, each G418-resistant clone was grown for 16 h in 6-well plates (5×10^3 cells/well) in 10% FBS with DMEM medium, then shifted to Dulbecco's modified Eagle's medium with 10% FBS. After 12-h incubation, pTRE-CT105 (1.5 μg of DNA) was introduced by transfection with Lipofectin (Gibco Laboratories). After culturing in 10% FBS Dulbecco's modified Eagle's medium for 18 h and in 10% FBS RPMI medium for 48 h, the cells were incubated in 5.0 μg/ml doxycycline for 72 h to induce expression of rtTA protein. For cell survival, 1.5×10^4 cells per well were seeded in 96-well plate for 72 h, then switched to medium (Dulbecco's modified Eagle's medium containing 1% fetal bovine serum, doxycycline (5 μg/ml)) and NGF (50 ng/ml) on polylysine coat dish.

Cloned PC12 cells were seeded at 1×10^4 cells/ml in 96 well multiplate and were activated by incubation in medium containing doxycycline and various concentrations of test compounds dissolved in water or DMSO. At 72 h viable cells in each treatment group were estimated in a colorimetric assay that measures the formazan reduction product of MTT, which is produced by mitochondrial activity of viable cells. The reduction product was dissolved in a mixture of isopropanol, 0.1 N HCl, Triton X-100 (90 : 10 : 0.4), and absorbance was quantitated using a plate reader spectrophotometer. The net difference A_{570}/A_{650} was used to express the viability of the cells. Control experiments showed that the reduction of MTT was proportional to cell number. Images were photographed with an Olympus IX-70 microscope.

Statistical Analysis The data are expressed as the means±S.E., and statistical significance was determined by analysis of variance (ANOVA) with either Dunnett's test in the case of multiple comparisons with control. The differences were accepted as significant at $p<0.05$ and $p<0.01$.

Table 1. Effects of the Methanolic Extracts of Plants on the CT105-induced Neurotoxicity in PC12 Cells

Plant name	Cell survival (%)[a]
Control	100±1.5
CT105	18.5±1.5
Polygala tenuifolia	45.6±2.5
Acorus gramineus Solander	52.7±1.3
Uncariae Ramulus et Uncus	83.7±4.2
Gastrodia elata	78.5±3.6
Angelica gigas	48.1±2.8
Evodia officinalis	59.2±3.5
Panax ginseng	69.5±1.1
Cnidium officinalis	34.2±4.2
Eucommia ulmoides	32.4±6.0
Acanthopanacis Cortex Radicis	45.6±1.7

a) Final concentrations of samples in culture media were 50 μg/ml PC12 cells were induced with CT_{105} for 72 h, together with one of ten different plant extracts, and the degree of cell survival was assessed using MTT assay. The values are the means from the triplicate experiment. The values indicate the degree of cell survival expressed as a % of the control level (mean±S.E.M).

Table 2. Survival Effect of the Various Fractions of Plant Extracts on the CT105-Induced Neurotoxicity in PC12 Cells

Plant name	Solvent fra.	Cell survival (IC_{50})[a]
Uncariae Ramulus et Uncus	Ether	35.1
	EtOAc	14.2
	BuOH	4.8
Gastrodia elata	Ether	38.5
	EtOAc	24.2
	BuOH	8.3
Evodia officinalis	Ether	43.4
	EtOAc	32.1
	BuOH	15.6
Panax ginseng	Ether	36.0
	EtOAc	19.7
	BuOH	11.2

a) Final concentration of samples in culture media in μg/ml. PC12 cells were induced with CT_{105} for 72 h, together with one of 12 different plant extracts, and the degree of cell survival was assessed using MTT assay. The values are the means from the triplicate experiment.

RESULTS AND DISCUSSION

Following induction of CT_{105} for 72 h, cell survival was reduced to 18.5±1.5% compared to the control. Of the 10 methanol extracts that were treated against CT_{105}-induced PC12 cells, *Uncariae Ramulus et Uncus* (UREU), *Gastrodia elata* (GAE), *Evodia officinalis* (EO) and *Panax ginseng* (PAG) showed enhancement of cell survival compared to the CT_{105}-induced PC12 cell group. The enhancement of cell survival compared to control level amounts to *ca.* 54% blockade of CT_{105}-induced cell death. The degrees of cell survival ranged from 59.2±3.5% to 83.7±4.2% of the control level (40.7—65.2% blockade of the CT_{105}-induced cell death). This is comparable to the level of cell protection by estrogen (*ca.* 36% blockade of Aβ-induced cell death) under a similar experimental condition.[16] Table 1 shows the effects all 10 plants extract on CT_{105}-induced PC12 cell death.

We further tested the effects of twelve solvent fractions isolated from the four methanol extracts that showed substantial protective effects against CT_{105}-induced cell cytotoxicity. Toxicity was induced by the expression of CT_{105} in PC12 cells for 72 h. Of the twelve solvent tested, only two enhanced cell survival compared to the CT_{105}-induced group. The IC_{50} value of BuOH fraction from UREU and GAE are significantly determined 4.8 μg/ml and 8.3 μg/ml about cell survival, respectively. These effects of the twelve solvent fractions on CT_{105}-induced cell cytotoxicity are shown in Table 2. All four BuOH fractions that showed protective effects against CT_{105} toxicity enhanced cell survival (Fig. 1). The degrees of cell survival were between 52.7±1.3% and 83.7±4.2% of the control level. Morphologically, CT105-induced neurotoxicity manifested as early shrinkage and late loss of cell bodies (Fig. 2); BuOH fraction of UREU and GAE regained many neurons from CT105 neurotoxicity.

The main aim of the present study was to test whether fractions isolated from UREU and GAE protect neurons against CT_{105}-induced PC12 cells toxicity. Among of the 10 tested methanol extract of several plants, *Uncariae Ramulus et Uncus, Gastrodia elata, Evodia officinalis* and *Panax ginseng* significantly protected PC12 cells from CT_{105}-induced cell death in a considerable manner (enhancement > con-

trol). The nature of CT_{105}-induced cell death is yet not clear. Previous studies suggest that apoptotic cell death, oxidative stress and necrotic cell death are all involved in Aβ-induced cell death.[17,18] Recently, it has been reported that danthron, a component of *Rumex japonicus*, senna, and aloe, attenuates β-amyloid-induced neurotoxicity in a murine cortical culture system.[19—21] This study, we examined the cytotoxicity and antiapoptotic effects of the four BuOH fraction that significantly protected neurons from CT_{105} toxicity. The results show that all of them had the protective effects against cell death, whereas two fractions of UREU and GAE had additional antidementia effects. Thus, the neuroprotective effects of the two fractions against CT_{105} toxicity are probably based on both protective effect against CT_{105}-induced PC12 cell toxicity and cell survival effects, but antiapoptotic effects play a more important role.

The neuroprotective effects of the ten plant extracts were evalulated by the MTT assay measuring the viability in CT_{105}-induced PC12 cells. Among these twelve solvent fractions, only two BuOH fractions significantly attenuated neuronal cell death induced by CT_{105} expression in PC12 cells. BuOH fraction of UREU and GAE was found to exhibit potent neuroprotective effects at a concentration of 4.8 and 8.3 μg/ml (IC_{50}), respectively.

Our finding that BuOH fraction of UREU and GAE protects CT_{105}-induced cytotoxicity in PC12 cells may thus suggest that the fractions can promote neuroprotective effects in neuronal cells. In conclusion, antiapoptotic effects of the two fractions that protected neurons from CT105-induced neuronal cytotoxicity. Further studies are needed to clarify the neuropotective effect of the fractions *in vivo*.

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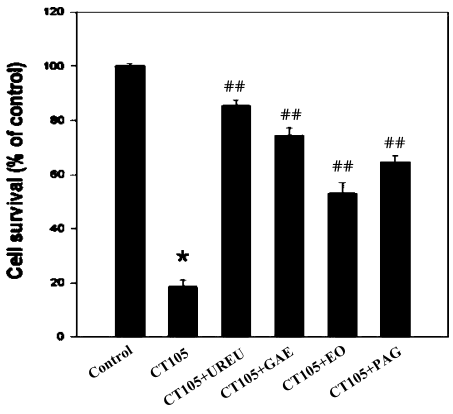

Fig. 1. Effects of Isolated Solvent Fraction on CT_{105}-induced Cell Survival

CT_{105}-induced PC12 cells were treated with one of four plant extracts for 12 h that showed substantial protective effects against CT_{105} toxicity, and the degree of cell survival expressed as a % of the control level. The degree of cell survival was assessed using MTT assay. The untreated control was set to 100%. All of the data, expressed as percentages of control, were the means±S.E. of three separate experiments. $*p<0.05$; *versus* control group, $\#\#p<0.01$; *versus* CT_{105}-induced group (ANOVA and Dunnett's test).

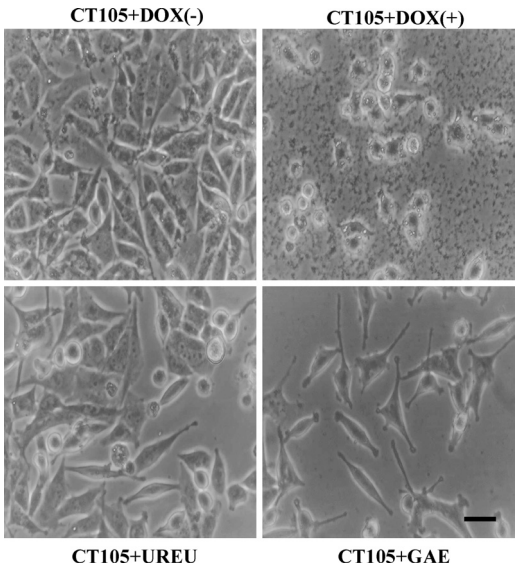

Fig. 2. Phase Contrast Photomicrographs Depicting Representative Morphological Responses of CT_{105}-Induced PC12 Cells by the BuOH Fraction of UREU and GAE

PC12 cells were observed for 72 h in the without (upper left) or with doxycycline (5 μg/ml) (upper right) for CT_{105} induction, and treated with UREU (lower left) or GAE (lower right) under CT_{105} induction, respectively. Microscopy analysis shown with cell death and detached or loosely adhered rounded cells at 24 h for CT_{105}-induced cells. (Magnification, ×200). CT_{105}-induced PC12 cells were observed with phase-contrast inverted microscopy (×200). The experiment was performed twice with similar results. DOX represented as doxycycline for CT_{105} inducer. Bars indicates 50 μm.

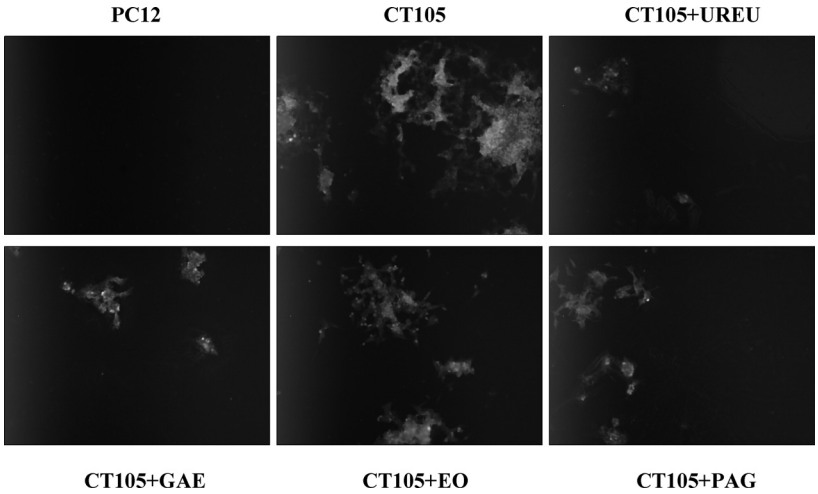

Fig. 3. The Inhibitory Effect of Treatment with Various Fractions on CT_{105}-Induced Apoptosis in PC12 Cells

Fluorescence microscopy studies were done as APO-BrdUrd Tunel assay. Apo-BrdUrd (TUNEL) assay for DNA strand breaks (late apoptosis). Fluorescence image reflects DNA fragmentation. The experiment was performed twice with similar results. CT_{105}-induced PC12 cells were observed with Fluorescence microscopy (×100).

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