Protective Effects of Toki-Shakuyaku-San Tsumra Japan-23 (TJ-23) on β-Amyloid Protein (β40)-induced Apoptosis in Pheochromocytoma-12 (PC12) Cells

Yuan Ying Liu*,[a,b] and Takanobu Kojima[a]

[a]*Department of Traditional Chinese Medicine, Faculty of Pharmaceutical Sciences and* [b]*Organization for Frontier Research in Preventive Pharmaceutical Sciences, Hokuriku University, Ho–3, Kanagawa-machi, Kanazawa, Ishikawa 920–1181, Japan*

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The protective effect of a Japanese herbal medicine, Toki-Shakuyaku-San extract (TJ-23) was investigated on β-Amyloid protein (β40)-induced apoptosis in PC12 cells. TJ-23 has been reported to activate cholinergic neurons in the brain, and to aid in the recovery from the spatial cognition disorder induced by scopolamine in rats. The association between neuron death in Alzheimer's disease (AD) and apoptosis has attracted attention, and studies in cultured cells have suggested that β-Amyloid protein induces cell death by apoptosis. The pathway for the induction of apoptosis is caspase cascade activation. In addition, caspase-3 activation due to β-induced injury in PC12 cells has been reported. We evaluated the caspase-3 activity of TJ-23 on β-induced apoptosis in PC12 cells using a fluorophotometer. In our study, TJ-23 significantly inhibited the increase in lactate dehydrogenase (LDH) release following β40-induced cell injury and significantly increased 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction, significantly increasing the cell survival rate. These results suggested the protective effects of TJ-23. In addition, the inhibition of β40-induced cell injury and the significant increase in the cell survival rate by TJ-23 were continuous, suggesting continuous protective effects. TJ-23 significantly inhibited caspase-3 activation due to β40-induced cell injury. These results suggest that a pathway via caspase-3 activation is one of the mechanisms of the protective effects of TJ-23.

Key words —— Toki-shakuyaku-san (Tsumra Japan-23), β-amyloid protein (β40), Alzheimer's disease, caspase-3, Pheochromocytoma-12 cell

INTRODUCTION

Toki-shakuyaku-san (Tsumra Japan-23 (TJ-23)) is a Kampo medicine obtained after hot water extraction of angelicae radix, paeoniae radix, cnidium rhizome, atractylodes lancea rhizome, alisma rhizome, and hoelen, followed by drying and mixing. TJ-23, which promotes estrogen secretion, is widely used for diseases in the gynecological field such as ovarian insufficiency, sterility, irregular menstruation, and climacteric disturbance. In climacteric disturbance, estrogen secretion decreases, and the incidence of Alzheimer's disease (AD) is high; therefore, the association between estrogen and dementia has been suggested, and estrogen therapy is performed for AD in the U.S. TJ-23, which promotes estrogen secretion, has fewer adverse effects than tacrine or donepezil. Other studies have shown an increase in nicotinic Ach receptor binding ability in the cerebral cortex after a 1 week administration of TJ-23 to 3 week old rats,[1] and an increase in choline acethyltransferase activity in the brain after a 2 week administration of TJ-23 to 5 month old female rats.[2] TJ-23 has been reported to activate cholinergic neurons in the rat brain and to enhance recover from the disorder of spatial cognition induced by scopolamine in rats.[3–5]

These observations suggest that TJ-23 may protect against the dementia induced by the dysfunction of cholinergic neurons. A recent study showed that the administration of TJ-23 to rats that underwent scopolamine administration significantly improved spatial cognition disorder. With the aging of society, patients with AD or vascular dementia have rapidly been increasing; therefore, clarification of

*To whom correspondence should be addressed: Department of Traditional Chinese Medicine, Faculty of Pharmaceutical Sciences, Hokuriku University, Ho–3, Kanagawa-machi, Kanazawa, Ishikawa 920–1181, Japan. Tel.: +81-76-229-6228; Fax: +81-76-229-6228; E-mail: y-liu@hokuriku-u.ac.jp

the mechanism of age-related development of dementia and the development of drugs useful for its prevention and treatment are eagerly awaited. We therefore investigated the neuroactivity of TJ-23 in terms of its ability to protect cultured Pheochromocytoma (PC)12 cells, a clone of rat pheochromocytoma cells, from the lethal effect of β40 and its mechanism.

MATERIALS AND METHODS

Cell Cultures and Treatment with β40 or Drugs —— PC12 cells derived from rat pheochromocytoma of the adrenal gland were cultured on 35 mm dishes or 48 well microplates coated with 0.01% poly-d-lysine at a CO_2 concentration of 5% at 37°C for 24 hr. The cell density was 5×10^4 or 5×10^5 cells/ml. β40 (Wako, Osaka, Japan: purchased from the Peptide Institution Inc.) was suspended in CMF-PBS, aged for 7 days, and ultrasonicated for uniform dispersion of aggregates immediately before its addition to the cell culture. Toki-shakuyaku-san extract (TJ-23, Tumura & Co., Tokyo, Japan) was dissolved in serum-free medium. One hour after TJ-23 administration, β40 (10–20 μM) was added to the culture system.

Methods for Assay of Cell Viability —— Two biochemical methods, determination of the lactate dehydrogenase (LDH) release and activity of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction of cells, were used. To determine the activity of LDH released in medium, 0.25 ml of medium was subjected to the conventional rate assay of absorbance at 340 nm for 1 min with a spectrophotometer (U-2000, HITACHI, Tokyo, Japan) as described previously for each well. Data are expressed as $\Delta A/min \times 10^3$. For the MTT reduction assay,[6] 20 μl of MTT solution in CMF-PBS at (2.75 mg/ml was added to each well on removal of the medium sample for LDH assay. The plates were incubated in a CO_2 concentration of 5% at 37°C for 30 min. Absorption at 550 nm of the solution was measured with a microplate reader (Immuno-Mini NJ-2300; Nippon InterMed, Tokyo, Japan) using 96-well plates.

Caspase-3 Activity Assay[7,8] —— At 24 hr and 48 hr treatment of β40 in 35 mm dishes after culture for selection, as described for cells of culture plates, each dish of cell layers was rinsed with cold CMF-PBS and harvested with a rubber policeman into 250 μl of lysis buffer to homogenize in an injection syringe with 25G needle. Each cell homogenate was sonicated and centrifuged for 30 min at 13000 rpm at 4°C. Samples of the supernatant (30 μg protein per sample) were incubated with a fluorogenic substrate, N-acetyl-Asp-Glu-Val-Asp-(Ac-DEVD-)7-amino-4-methylcoumarin (AMC) (20 μM), specific for caspase-3 at 37°C for 1 hr. Ac-DEVD-AMC was purchased from the Peptide Institute and dissolved in dimethyl sulfoxide (DMSO) at 10 mM. The reaction was stopped by the addition of 2.5 nM monoiodoacetic acid. Cleavage of the substrate by caspase-3 was measured by the fluorescence of the product, AMC, using a Hitachi 850 fluorescence spectrophotometer (excitation at 380 nm and emission at 460 nm). The protein content of the samples for various assays was determined according to the method of Lowry *et al.*[8] using bovine serum albumin as standard.

Statistical Analysis —— Results of experiments were expressed as the mean (n = 5–7) ± S.D. Statistical differences between results were analyzed by Student's t-test.

RESULTS

Morphological Changes in PC12 Cells After Drug Administration

The actions of TJ-23 on PC12 cells were observed in terms of morphological changes under a phase contrast microscope. The morphology of PC12 cells 48 hr after the administration of 50 μg/ml TJ-23 was similar to that of the control (Fig. 1). These results suggested that TJ-23 causes no injury in PC12 cells. The morphology of PC12 cells 48 hr after the addition of Aβ40 (10–20 μM) following the administration of TJ-23 (50 μg/ml) was observed using a phase contrast microscope. In the coexistence of TJ-23, cell atrophy decreased (Fig. 1); therefore, the effects of TJ-23 on Aβ40-induced cell injury were evaluated.

Actions of TJ-23 on PC12 Cells

LDH release significantly decreased after TJ-23 administration alone to 87% and 64% as compared with control values for 24 and 48 hr, respectively (Fig. 2). This decrease was time-dependent. The significant time-dependent decrease in LDH release after TJ-23 administration alone suggested the protection of PC12 cells by TJ-23.

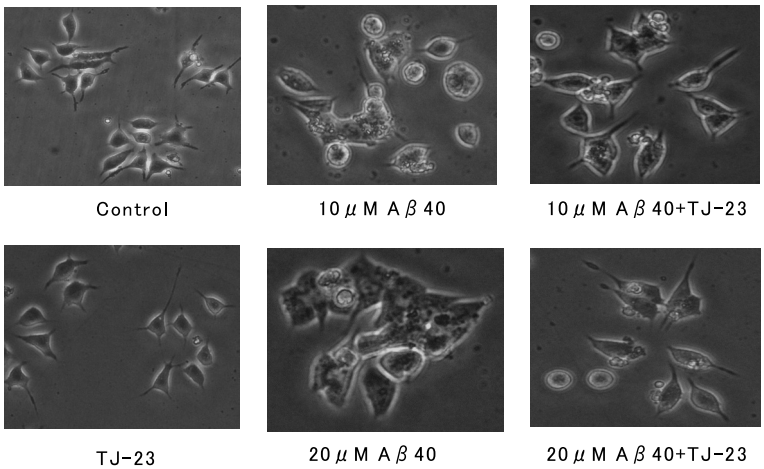

Fig. 1. Morphological Changes in PC12 Cells after Drug Administration.

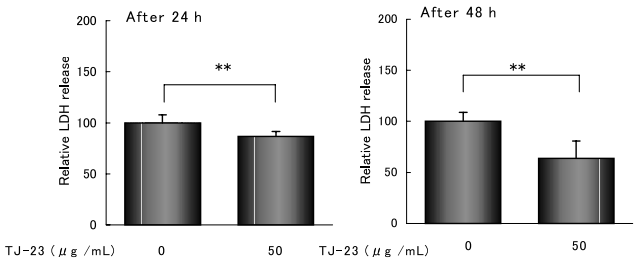

Fig. 2. Effect of TJ-23 on PC12 Cells. PC12 Cells were Incubated with TJ-23 for 24–48 hr. LDH Release was Determined as the Effect of TJ-23 and Expressed as % of Control as Described in Materials and Methods.

Values represent the mean ± S.D. (n = 7) of LDH release as % of control. $^{**}p < 0.01$ *vs.* control Student's *t*-test.

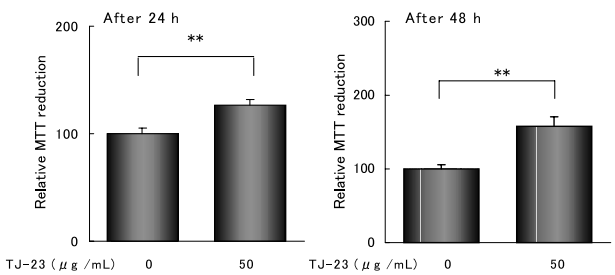

Fig. 3. Effect of TJ-23 on PC12 Cells. PC12 Cells were Incubated with TJ-23 for 24–48 hr. MTT Reduction was Determined as the Effect of TJ-23 and Expressed as % of Control as Described in Materials and Methods.

Values represent the mean ± S.D. (n = 6) of MTT reduction as % of control. $^{**}p < 0.01$ *vs.* control. Student's *t*-test.

MTT reduction significantly increased after TJ-23 administration alone to 127% and 158%, as compared with control values for 24 and 48 hr, respectively (Fig. 3).

These results suggested a time-dependent increase of the surviving cell count after TJ-23 administration. The significant time-dependent increase in MTT reduction after TJ-23 administration alone suggests an increase in the surviving cell count. TJ-23 may promote PC12 cell proliferation. TJ-23 itself did not affect the cell viability of PC12 cells. These results suggest that TJ-23 has continuous protective effects on PC12 cells, promoting cell proliferation.

TJ-23 Prevents β40-induced Apoptosis of PC12 Cells

The protective effects of TJ-23 were evaluated by MTT reduction and LDH release assays of β40-induced cell death of PC12 cells. Ten micromoles of β40-induced a 262% and 209% increase of LDH release as compared with control values for 24 and 48 hr, respectively. Twenty micromoles of β40-induced a 236% and 257% increase of LDH release as compared with control values for 24 and 48 hr, respectively (Figs. 4, 5); these increases appeared to be time-dependent. MTT reduction significantly decreased after the addition of 10 μM and 20 μM β40 to 33.5%, 14.3% and 26.6%, 18.6% as compared with control values for 24 and 48 hr, respectively (Figs. 4, 5). This suggests that β40 time-dependently decreases the cell survival rate. In the presence of TJ-23 (50 μg/ml), β40 neurotoxicity was dramatically suppressed in both assays. LDH release significantly decreased 24 and 48 hr after the addition of 10 μM β40 in the presence of TJ-23 to 131% and 116% as compared with control values for 24 and 48 hr, respectively. LDH release significantly decreased 24 and 48 hr after the addition of 20 μM β40 in the presence of TJ-23 to 146% and 120% as compared with control values for 24 and

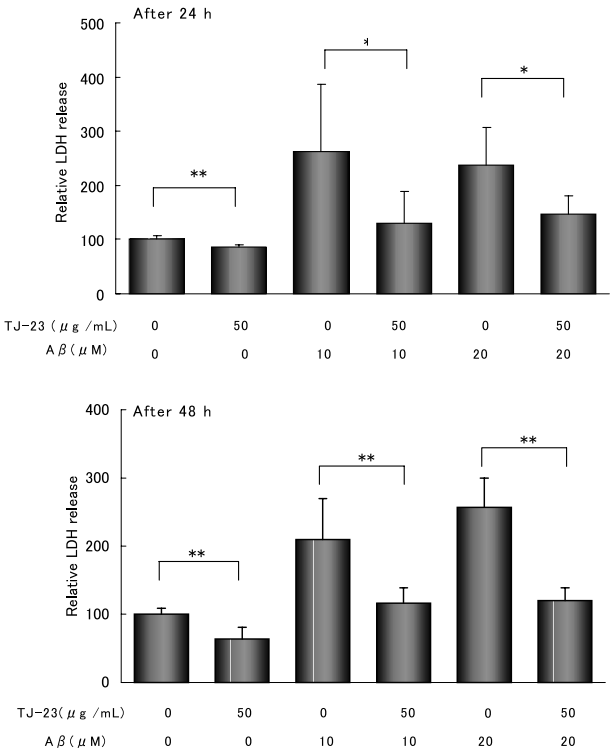

Fig. 4. The Protective Effect of TJ-23 on Aβ40-induced Cytotoxicity in PC12 Cells. PC12 Cells were Incubated with or without (Control and Aβ40 Only) TJ-23 for 1 hr and Additionally Incubated with or without (Control and TJ-23 Only) Aβ40 for 24–48 hr. LDH Release was Determined for the Evalution of Cytotoxicity and Expressed as % of Control as Described in Materials and Methods.

Values represent the mean ± S.D. (n = 7) of LDH release as % of control. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* control or Aβ40 only.

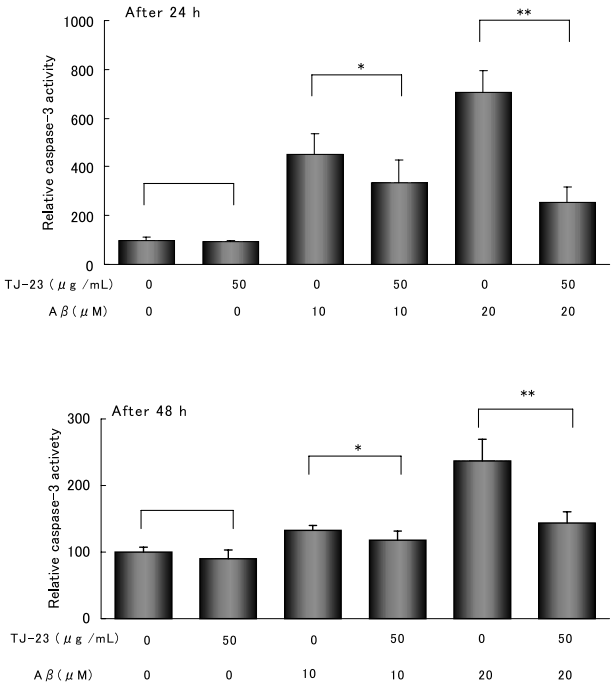

Fig. 5. Prevention of Aβ40-induced Caspase-3 Activation by TJ-23 in PC12 Cells. PC12 Cells were Incubated with or without (Control and Aβ40 Only) TJ-23 for 1 hr and Additionally Incubated with or without (Control and TJ-23 only) Aβ40 for 24–48 hr. PC12 Cells were Lysed with Lysis Buffer and 30 μg of Protein for Each Sample was Incubated with the Fluorogenic Substarate, Ac-DEVE-MCA (20 μM). Cleavage of the Substrate Emitted a Fluorescent Signal That was Measured Using a Fluorometer (Excitation at 380 nm, Emission at 460 nm). Details of the Method are Described in Materials and Methods.

Values represent the mean ± S.D. (n = 5–6) of caspase-3 activity as % of control. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* control or Aβ40 only. Student's t-test.

48 hr, respectively. MTT reduction significantly decreased after the addition of 10 μM β40 and 20 μM β40 in the presence of TJ-23 to 45.7%, 30.2% and 34.4%, 24.5% as compared with control values for 24 and 48 hr, respectively (Fig. 4). The cell survival rates 24 and 48 hr after the addition of 10 μM β40 in the presence of TJ-23 were 1.37 times and 2.11 times, respectively, those after the addition of 10 μM β40 alone. The cell survival rates 24 and 48 hr after the addition of 20 μM β40 in the presence of TJ-23 were 1.29 times and 1.32 times, respectively, those after addition of 20 μM β40 alone. In the presence of TJ-23, the cell survival rate continuously increased.

TJ-23 Prevents β40-induced Caspase-3 Activation of PC12 Cells

The activation of caspases is now well accepted to be responsible for apoptotic cell death (Matsuzawa and Ichijo, 2001). In addition, caspase-3 activation due to β-induced cell injury in hippocampal neurons has been reported. In particular, caspase-3 is a major activator of apoptotic signals. We therefore evaluated the β40 toxicity with a caspase-3 inhibitor of Ac-DEVD-AMC. Caspase-3 activities 24 or 48 hr after TJ-23 administration were similar to those in the control. Caspase-3 activity after the addition of 10 μM or 20 μM β40 significantly increased dose-dependently compared with the value in the control. Caspase-3 activity 24 or 48 hr after the addition of 10 μM or 20 μM β40 in the presence of TJ-23 significantly decreased compared with the addition of β40 alone (Fig. 5).

DISCUSSION

The role of amyloid protein in AD is not yet fully understood. Although it is clear that deposits

of insoluble amyloid protein are found in senile plaques in the brain of people with AD, particularly in the hippocampus, studies of primary and clonal neuronal cells with amyloid protein suggest two types of cell death: apoptosis (Forloni *et al.*, 1993; Loo *et al.*, 1993) and necrosis. Our present data demonstrated that PC12 cells underwent extensive apoptosis after treatment with β40. Basic studies have shown that TJ-23 reduces the glutaminic acid level in the cerebral cortex, hippocampus, and corpus striatum both in males and females. Clinical studies have shown improvement in motor function, cognitive disorder, and cerebrovascular sequelae after the administration of TJ-23 to patients with senile dementia, or improvement in motor function, intellectual function, emotional function and other psychiatric findings after its administration to AD patients. In our study, TJ-23 significantly inhibited the increase in LDH release following β40-induced cell injury and significantly increased MTT reduction, significantly increasing the cell survival rate. These results suggested the protective effects of TJ-23. In addition, the inhibition of β40-induced cell injury and the significant increase in the cell survival rate by TJ-23 were continuous, suggesting continuous protective effects. The association between neuron death in AD and apoptosis has attracted attention,[9] and studies in cultured cells have suggested that Aβ-induces cell death by apoptosis.[10] A pathway for the induction of apoptosis is caspase cascade activation. In addition, caspase-3 activation due to β40-induced injury in PC12 cells has been reported. To clarify the action mechanism of TJ-23 on β40-induced injury in PC12 cells, we evaluated the association with apoptosis and the involvement of TJ-23 by caspase-3 assay in a model of β40-induced cell injury. Caspase-3 activity in the cells incubated with β40 markedly increased compared with the control. These results suggested that β40 cell injury is mediated by caspase-3. Apoptosis is induced by caspase-3 activation. TJ-23 significantly inhibited caspase-3 activation due to β40-induced cell injury. These results suggest that a pathway via caspase-3 activation is one of the mechanisms of the protective effects of TJ-23.

Our results clarified one of the mechanisms of the protective effects of TJ-23 against β40-induced cell injury. We expect further clarification of the mechanisms of the protective effects of this medicine against β-induced cell injury in the future.

In this study, TJ-23 had significant inhibitory effects on cell injury and promoting effects on cell proliferation compared with the control, suggesting its protection and activation of cells; however, we speculate that mechanisms other than the pathway mediated by caspase-3 are involved in these effects.

TJ-23 significantly inhibited β40-induced cell injury and significantly increased the cell survival rate. These results suggest that this medicine inhibits caspase-3 activation and protects cells from β40-induced injury. Our results showed the protective effects of TJ-23 on PC12 cells and against β40-induced cell injury, suggesting that the mechanism of the protective effects is a pathway mediated by caspase-3.

TJ-23 has long been known to have effects on "blood deficiency/blood stasis/water poisoning" and is considered to be indicated for dementia, particularly deficiency syndrome. In this study, TJ-23 directly protected and activated PC12 cells and also had protective effects against β40-induced cell injury.

In this study, TJ-23 had significant protective effects against β40-induced cell injury, and new findings concerning its mechanism were obtained. This medicine may be useful for the prevention and treatment of AD and is also expected to inhibit neuron death associated with AD. Further studies are necessary.

In summary, the administration of TJ-23 increased the cell survival rate, which suggested its protective effects on neurons. TJ-23 significantly inhibited Aβ40-induced cell injury, which suggested its protective effects against neuron injury. TJ-23 inhibited Aβ40-induced neuron death, and a pathway associated with caspase-3 activity may be involved as one of the mechanisms of this protection.

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