Toki-Shakuyaku-San Intervenes in Apoptosis Induced by Lowering of Potassium Chloride Concentrations in Cultured Cerebellar Granule Cells†

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Cerebellar granular cells harvested from 8-day-old rats were cultured in a basic medium of Eagle's salt (BME) containing 10% serum and 25 mM KCl. At 7 days *in vitro* (DIV), the culture medium was switched to serum-free BME containing 5 mM KCl. Fluorescein diacetate/propidium iodide staining was used to examine the neuronal survival. Phase-contrast microscopy and DNA fragmentation with agarose gel were used for examination of apoptosis. A progressive neuronal death with nuclear condensation, extensive damage of the neuritis network, and DNA fragmentation were observed following a switch to 5 mM KCl BME. The neuronal survival was 64%, 48% and 30% at 24, 48 and 72 h following a switch to 5 mM KCl BME respectively.

An application of 0.05 mg/mL of Toki-Shakuyaku-San (TJ-23, a recipe of Japanese traditional medicine: Kampo medicine) to 5 mM KCl BME at 7 DIV intervened in neuronal death. The neuronal survival was 74% at 24 ($p < 0.05$), 72% at 48 h ($p < 0.001$) and 67% at 72 h ($p < 0.001$) following a switch to 5 mM KCl BME. An application of TJ-23 also intervened in nuclear condensation, DNA fragmentation, and damage of the neuritis network caused by a switch to 5 mM KCl BME. This study provides information that switching to 5 mM KCl BME from 25 mM KCl BME at 7 DIV causes apoptosis in the cultured cerebellar granule cells. Moreover, TJ-23 intervenes in apoptosis induced by a switch to 5 mM KCl BME in cultured cerebellar granule cells. © 1998 John Wiley & Sons, Ltd.

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There are two fundamental types of neuronal death: apoptosis and necrosis. Necrosis is initiated by cell membrane damage and it causes swelling of the cell (Buja *et al*., 1993). The morphological changes of apoptosis and characteristics of apoptosis have been well described in cultured sympathetic neurons following NGF deprivation (Edwards *et al*., 1991) and in cultured cerebellar granule neurons after lowering potassium chloride in the culture medium (D'Mello *et al*., 1993; Yan *et al*., 1994). There are also a few examples of neuronal apoptosis *in vivo* (Portera-Cailliau *et al*., 1995; Wood *et al*., 1993). However, the mechanism of apoptosis is still unclear. Apoptosis is believed to be critical for normal neuronal development (Wyllie *et al*., 1988), but there is increasing evidence that apoptosis may be triggered pathologically in the adult brain and it may be involved in non-physiological neuronal death (Martin *et al*., 1994; Portera-Cailliau *et al*., 1995; Smale *et al*., 1995; Cotman and Anderson, 1995). Loss of fully differentiated neurons should result in a critical loss of neuronal function and creates such degenerative neurological diseases as dementia of the Alzheimer type (DAT) (Zakeri and Lockshin, 1994; Martin *et al*., 1994; Hagino *et al*., 1995; Linnik *et al*., 1993). Therefore, medicines that intervene in apoptosis should be considered for the treatment of degenerative neurological diseases such as DAT (Schehr, 1994; Hagino, 1996).

Toki-Shakuyaku-San (TJ-23, Tsumura & Co. Tokyo, Japan) is a formula from the Kampo pharmacopoeia, which contains dried powder extract of the six medicinal plants: Paeony root 4.0 g; Atractylodes lancea rhizome japonica 4.0 g; Alisma rhizome 4.0 g; Hoelen 4.0 g; Cnidium rhizome 3.0 g; Angelica root 3.0 g. TJ-23 has been widely used for amenorrhoea, infertility and menopausal syndrome in China and Japan (Hagino, 1993). Recently, clinical application of TJ-23 to postmenopausal women with DAT has been shown to enhance their cognitive functions and to improve their sleep disturbance (Mizushima, 1989; Kudo and Sugiura, 1992; Itakura *et al*., 1992; Saito 1993; Fukushima *et al*., 1994; Inanaga *et al*., 1996). The results of clinical investigation and our previous study (Zhang, *et. al*., 1997) suggest that TJ-23 may intervene in apoptosis in the brain cells in DAT patients and it may improve the symptoms of DAT patients. Therefore, the study was

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designed to examine if TJ-23 intervenes in apoptosis induced by a switch to 5 mM KCl BME in cultured cerebellar granule cells.

MATERIAL AND METHODS

Primary neuron cultures. Cerebellar granule cells were prepared from 8-day postnatal Sprague-Dawley rats (Charles River, USA) and cultured as described by Gallo *et al*. (1982). Two mL of the cell suspension was plated in a 35-mm tissue culture dish precoated with poly-L-lysine (10 μg/mL) at a density of 2.5×10^6 cells/dish in basic medium of Eagle's salt (BME) containing 10% fetal calf serum, 2 mM glutamine, 50 μg/mL gentamycin and 25 mM KCl. Cultures were maintained at 37°C with 5% CO_2 in a humidified incubator. Cytosine arabinoside (final concentration 10 μM) was added 18 h after plating to arrest the growth of non-neuronal cells.

Treatment of cultures. Granule cells were cultured in 25 mM KCl BME containing 10% fetal calf serum for 7 DIV with no change of incubation medium. At 7 DIV the incubation medium was switched to serum-free 25 mM KCl BME or serum-free 5 mM KCl BME, with or without tested drugs.

Neurotoxicity and application of TJ-23. The neurotoxicity of TJ-23 and effective dosage of application of TJ-23 were discussed in a previous publication (Zhang *et al*. 1997): a dried powder extract of Toki-Shakuyaku-San (TJ-23 was supplied by Tsumura & Co., Tokyo, Japan) was dissolved in serum-free 5 mM KCl BME at a concentration of 10 mg/mL, and centrifuged at 3000 rpm for 30 min. The supernatant was filtered using 0.22 μm Corning filter units (New York, USA), and applied to the cultures at 7 DIV by dilution in 5 mM KCl BME. The filtered solution was applied to the cultures at 7 DIV by diluting in the corresponding incubation buffers (5 mM KCl BME) at 0.5 or 0.05 mg/mL. The neuronal damage was assayed with fluorescein diacetate (FDA) and propidium iodide (PI) stains. The concentration of KCl in the supernatant of the water extract of 0.05 mg/mL of TJ-23 was measured and only 0.05 mM of KCl was found in the solution. This would not alter the concentration of the 5 mM KCl medium.

Neuronal survival. At 24, 48 and 72 h after switching of the incubation media, the morphological changes were examined with phase-contrast microscopy, and the neuronal survival was examined by fluorescein diacetate (FDA) and propidium iodine (PI) stain as described by Jones and Senft (1985). Briefly, cultured cells were stained for 3 min at 22°C with Locke's solution containing 15 μg/mL of FDA and 80 μg/mL PI. The stained cells were then immediately examined with a standard epiillumination fluorescence microscopy (Vanox, Olympus; 450 excitation, 520 barrier). FDA crossed the cell membranes and was hydrolysed by intracellular esterase to produce green-yellow staining; PI interacted with DNA to yield red fluorescence. The surviving percentage was calculated by assessing the ratio between the FDA and FDA plus PI staining in photomicrographs. More than 500 cells were counted in each photomicrograph, with three photomicrographs for each sister culture. The photos of microscopy stained by FDA/PI were presented previously (Zhang, *et al*., 1997).

DNA fragmentation analysis. Cells were harvested at 24, 48 and 72 h following a switch to a testing incubation media. The genomic DNA was extracted as follows: cells (2.5×10^6) were lysed in 1 mL of extraction buffer [10 mM Tris (pH 8),100 mM EDTA,0.5% sodium dodecyl sulphate and 20 μg/mL of RNase A] for 1 h at 37°C and incubated further for 3 h at 50°C after the addition of proteinase K (100 μg/mL). Genomic DNA was sequentially extracted with an equal volume of phenol (pH 8), phenol/chloroform (1:1) and chloroform, precipitated with ammonium acetate (7.5 M; 0.5 vol) and ethanol (100%; 2 vol), and recovered by centrifugation. The DNA was re-suspended in 10 mM Tris-HCl and 1 mM EDTA and its concentration was determined by UV absorbancy at 260 and 280 nm. Equal amounts of DNA were then loaded onto 1.2% agrose gel containing ethidium bromide (0.5 μg/mL). After electrophoresis, DNA was visualized by ultraviolet illumination and photographed.

Statistics. Values are means ± SD from 3 to 4 separate experiments; three or more dishes were used in each experiment. The data were compared by Anova Fisher test.

RESULTS

Neurotoxicity of fresh serum

Cerebellar granule cells cultured in a 25 mM KCl BME containing 10% fetal calf serum showed that 91% ± 5.0% of neurons survived at 7 DIV. The surviving cells gave bright green-yellow fluorescence (FAD positive) with a rich network of neuritis against a dark background photos of microscopy stained by FDA/PI were presented previously (Zhang *et al*., 1997). Under phase-contrast microscopy, these cells were healthy with round cell bodies, clear nucleus and strong neuritis networks. When the culture medium was switched to a fresh 25 mM KCl BME containing a 10% fetal calf serum at 7 DIV, a significant neuronal death was observed. The neuronal survival was 64% ± 2.6%, 59% ± 2.5% and 55% ± 3.0% at 24, 48 and 72 h following a switch to a fresh medium with 10% calf serum respectively (Fig. 1), however, no DNA fragmentation was observed. When the medium was switched to a fresh serum-free 25 mM KCl BME at 7 DIV, the neuronal survival was 89% ± 4.3%, 89% ± 4.1% and 88% ± 3.1% at 24, 48 and 72 h following a switch to a fresh serum-free medium respectively (Fig. 1).

Therefore, cerebellar granule cells were cultured in a 25 mM KCl BME containing 10% fetal calf serum for 6 days and on the following day (7 DIV) the culture medium was switched to a serum-free BME to avoid the neurotoxicity of fresh serum.

Neuronal death following switch to 5 mM KCl BME at 7 DIV

Figure 1 shows the time course of neuronal death

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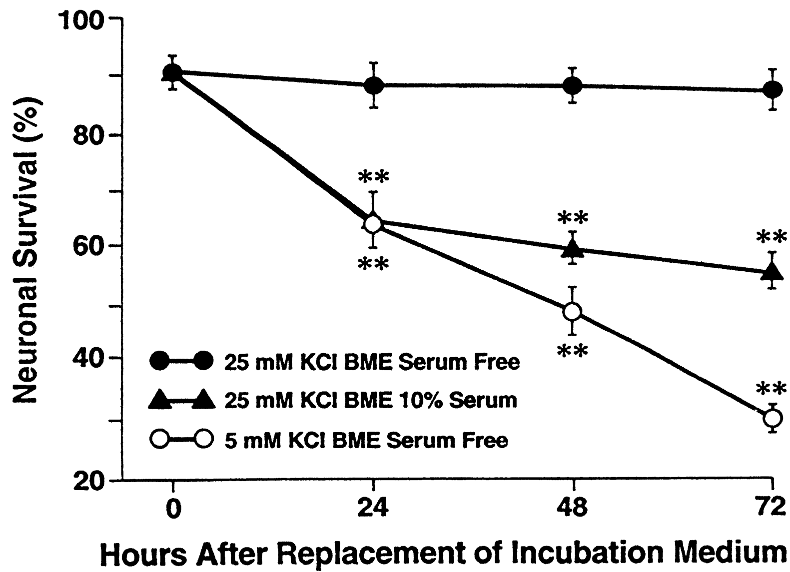

Figure 1. Neuronal survival after the replacement of the incubation medium at 7DIV. The replacement of the incubation medium with fresh 25 mM KCl BME containing 10% fetal bovine serum caused a significant neuronal loss. Meanwhile, the replacement of the incubation medium with serum-free 25 mM KCl BME did not induce neuronal death. However, the replacement of the incubation medium with serum-free 5 mM KCl BME caused a rapid and progressive neuronal death (apoptosis). Data are mean ± SD, four dishes for each experiment, 3 to 4 separate experiments. Significance was examined using Anova Fisher test. * $p < 0.05$; ** $p < 0.001$ compared with serum free 25 mM KCl BME control.

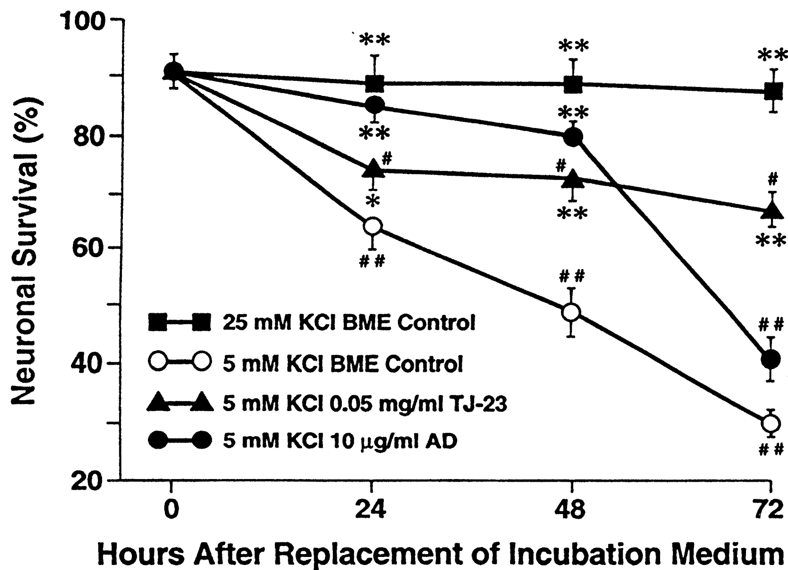

Figure 2. Neuronal survivals of either Toki-Shakuyaku-San(TJ-23) or Actinomycin(AD) treated cultures after the replacement of the incubation medium with serum-free 5 mM KCl BME. An application of 0.05 mg/mL of TJ-23 inhibited the apoptosis induced by the replacement of the incubation medium with serum-free 5 mM KCl BME significantly. Meanwhile 10 μM of AD inhibited the neuronal death at 24 and 48 h following the replacement of the incubation medium with serum-free 5 mM KCl BME. However, a significant neuronal death was observed at 72 h after cells were exposed to 10 μM of AD. Data were mean ± SD, four dishes for each experiment, 3 to 4 separate experiments. Significance was examined using Anova Fisher test. * $p < 0.05$; ** $p < 0.001$ comparing with serum-free 5 mM KCl BME control 2; # $p < 0.05$, ## $p < 0.001$ comparing with serum-free 25 mM KCl BME control 1.

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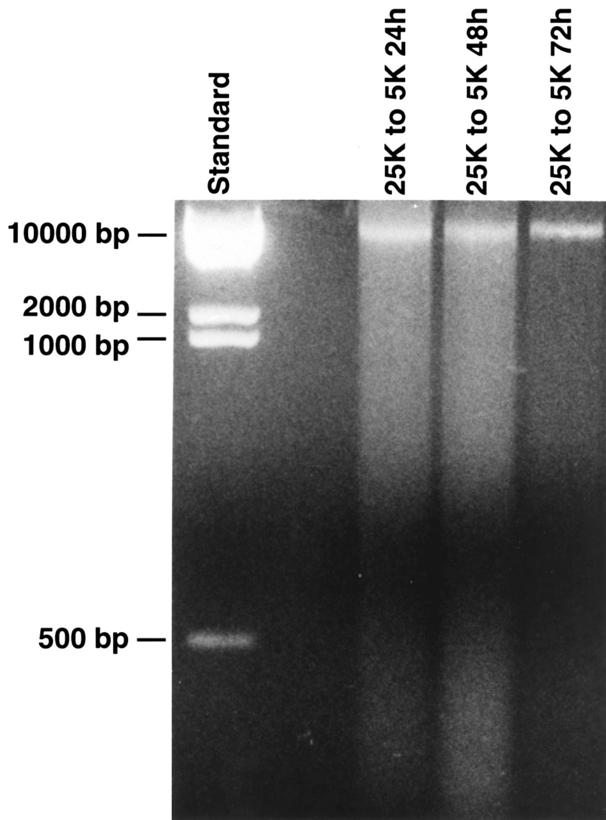

Figure 3. Replacement of the incubation medium with serum-free 5 mM KCl BME induced DNA fragmentation. Genomic DNA was extracted 24, 48 and 72 h after the replacement of the incubation medium and run on 1.2% agarose gel.

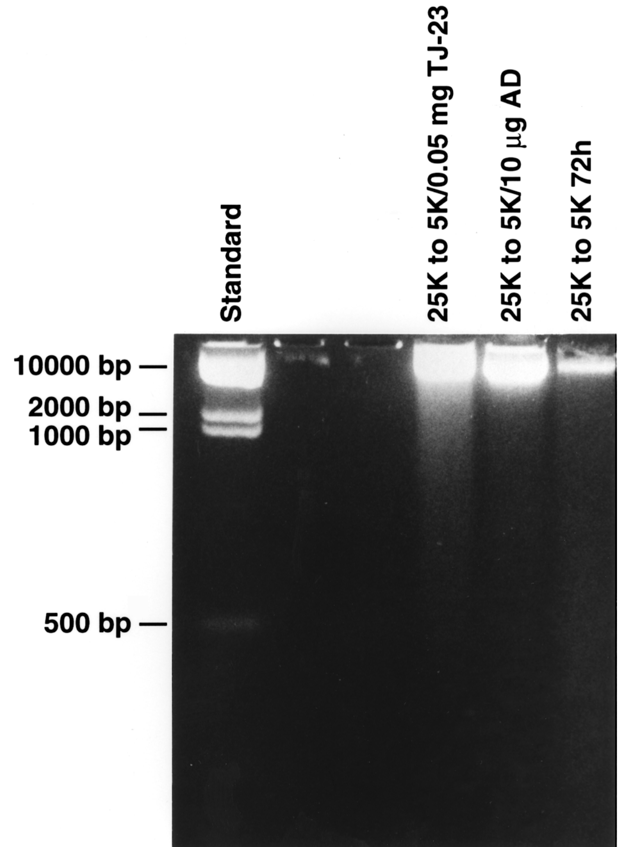

Figure 4. Both TJ-23 and AD intervened in DNA fragmentation induced by the replacement of the incubation medium with serum-free 5 mM KCl BME significantly. Genomic DNA was extracted 72 h after the replacement of the incubation medium and run on 1.2% agarose gel.

following a switch to a serum-free 5 mM KCl BME at 7 DIV. The neuronal survival was 64% ± 1.8% ($p < 0.05$) at 24 h following a switch to a serum-free 5 mM KCl BME, and the FDA positive neurons were shrunken and had lost the bright appearance of illumination fluorescence microscopy. Vacuoles in the neurons, condensation of nuclei and damage of the neuritis network were observed by phase-contrast microscope. DNA fragments were detected clearly by agarose gel electrophoresis at this time (Fig. 3: 25K to 5K 24 h). At 48 h following a switch to a 5 mM KCl BME, the neuronal survival was 48% ± 3.8% ($p < 0.001$), and the nuclear condensations became apparent and damage to the neuritis network was extensive. DNA fragmentation was detected clearly (Fig. 3: 25K to 5K 48 h). At 72 h following a switch to 5 mM KCl BME, the neuronal survival was only 30% ± 2.9% ($p < 0.001$), and the surviving cells were stained weakly with FDA. The neuritis network was damaged extensively and DNA fragmentation became prominent (Fig. 3: 25K to 5K 72 h).

Neuroprotective effects of Toki-Shakuyaku-San(TJ-23) on neuronal death following switch to 5 mM KCl BME at 7 DIV

Figure 2 shows the time course of neuronal death in the cultured cells treated with 0.05 mg/mL of TJ-23. An application of 0.05 mg/mL of TJ-23 to serum-free 5 mM KCl BME at 7 DIV intervened in the neuronal death: neuronal survival was 74% ± 3.5% ($p < 0.01$), 72% ± 3.9% ($p < 0.001$), and 67% ± 3.6% ($p < 0.001$) at 24, 48, and 72 h following a switch to 5 mM KCl BME. There were 52% ± 4.5% surviving neurons at 96 h following treatment with 0.05 mg/mL TJ-23 at 7 DIV.

TJ-23 treated cells were healthy with round cell bodies and showed a bright FDA staining with a strong neuritis network. TJ-23 also intervened in DNA fragmentation induced by switching the medium to a serum-free 5 mM KCl BME (Fig. 3: 25 K to 5 K/0.05 mg TJ-23).

Neuroprotective effects of actinomycin D(AD) on neuronal death following a switch to 5 mM KCl BME at 7 DIV

Figure 2 also shows the time course of neuronal death of cultured cerebellar granule cells treated with 10 μg/mL of AD. An application of 10 μg/mL of AD for the cultured cerebellar granule cells following a switch to a serum-free 5 mM KCl BME at 7 DIV intervened in neuronal death for 48 h; the neuronal survival was 85% ± 3.0% ($p < 0.001$) and 80% ± 2.5% ($p < 0.001$) at 24 and 48 h respectively following a switch to 5 mM KCl BME. The AD-treated cells were healthy with round cell bodies and showed a bright FDA staining with a strong neuritis network. However, the AD-treated cells exhibited neuronal swelling and extensive damage of the neuritis network at 72 h following a switch to 5 mM KCl BME. Only 41% ± 4.0% of the neurons survived (Fig. 2).

Moreover, the cultured cells treated with 10 μg/mL of

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AD at 7 DIV did not show the nuclear condensation and DNA fragmentation at 24, 48 and 72 h following a switch to 5 mM KCl BME (Fig. 4: 25 K to 5 K/10 μg AD). All the cultured cells were dead at 96 h following exposure to 10 μg/mL of AD at 7 DIV.

DISCUSSION

Cerebellar granule cells which were harvested from early postnatal rats grew in a culture medium containing serum with elevated levels of potassium chloride (25 mM KCl). These cells differentiated acquiring the morphological, biochemical and electrophysiological characteristics of mature neurons (Segal *et al*., 1992; Burgoyne and Cambray-Deakin, 1988; Kharlamov *et al*., 1995; Shambaugh III *et al*., 1994). In our study, it was observed that the cultured cerebellar granule cells became matured neurons at 7 DIV in 25 mM KCl BME with 10% calf serum, however, neuronal death occurred progressively; showing nuclear condensation, extensive damage of neuritis networks and DNA fragmentation following a switch to a serum-free 5 mM KCl BME from 25 mM KCl BME at 7 DIV (Figs 1, 3). DNA fragmentation was clearly observed at 8 h following a switch to a serum-free 5 mM KCl BME, even though the neuronal survival rates (FDA positive neurons) were 85% $\pm$ 4.6% at that time (data not shown). At 24 h following a switch to 5 mM KCl BME, 64% of the neurons survived (FDA positive neurons) (Fig. 2), but the DNA fragmentation became prominent (Fig. 3). These data demonstrate that the cleavage of DNA into oligonucleosomal size fragmentation was an early event in the death processes of neurons following a switch to a serum-free 5 mM KCl BME and appears prior to nuclear condensation. It is concluded that a switch to 5 mM KCl BME from 25 mM KCl BME during the culture induced apoptosis in the cerebellar granule cells.

Our results agree with the results of other investigators (D'Mello *et al*., 1993; Yan *et al*., 1994) confirming that cultured cerebellar granule cells in low potassium chloride were a suitable neuronal model for a study of the therapeutic efficacy of agents for intervention in apoptosis. Therefore, we used this model to study the therapeutic efficacy of TJ-23 for treatment of apoptosis following a switch to a serum-free 5 mM KCl BME from 25 mM KCl BME with 10% of calf serum in the cultured cerebellar granule cells at 7 DIV. It was observed that treatment with 0.05 mg/mL of TJ-23 at 7 DIV intervened in the neuronal death and damage of the neuritis network following a switch to 5 mM KCl BME from 25 mM KCl BME with 10% calf serum in the cultured cerebellar granule cells (Fig. 2). Moreover, treatment with 0.05 mg/mL of TJ-23 also intervened in DNA fragmentation caused by a switch to a serum-free 5 mM KCl in the cultured cerebellar granule cells (Fig. 4). The effective dosage of TJ-23 was discussed in a previous publication (Zhang *et al*., 1997): A dose of 0.05 mg/mL of TJ-23 was chosen for use as the plateau dose with 0.05–0.5 mg/mL causing the same effect.

It is possible that treatment with TJ-23 may intervene in apoptosis by way of the inhibition of 'killer gene' transcription. In order to examine this working hypothesis, the cultured cerebellar granule cells were treated with 10 μg/mL of actinomycin D(AD) after the culture medium was switched to 5 mM KCl BME at 7 DIV. It was found that an application of 10 μg/mL of AD to the cultured cerebellar granule cells intervened in neuronal death, the damage of the neuritis network, and DNA fragmentation at 24 and 48 h following a switch to 5 mM KCl BME (Figs 2, 4). From the observation made in this study, it seems likely that a switch to 5 mM KCl BME at 7 DIV may facilitate macromolecule synthesis (Martin *et al*., 1988; Villa *et al*., 1994) and thus may induce apoptosis in cultured cerebellar granule cells. However, we observed 60% neuronal death at 72 h after cerebellar granule cells were exposed to 10 μg/mL of AD (Figs 2, 3), and further, those cells showed extensive swelling of the cell body and loss of the neuritis network. However, these cells did not show the nuclear condensation and DNA fragmentation (Fig. 4). Treatment with AD intervened in apoptosis of cultured cerebellar granule cells up to 48 h following a switch to a serum-free 5mM KCl BME, however, 60% of cultured cells demonstrated neuronal death at 72 h after treatment with AD. Moreover, all cells were dead at 96 h after cerebellar granule cells were exposed to 10 μg/mL of AD. These data provide information that the neuronal death of cultured cerebellar granule cells at 72 h after exposure to 10 μg/mL AD was necrosis and not apoptosis. It may well be that AD would intervene in apoptosis by way of inhibition of 'killer gene' transcription, but AD would also inhibit the synthesis of RNAs and the following protein synthesis which are essential for neuron survival.

The survival of cells which were treated with 0.05 mg/mL of TJ-23 was less than that of cells which were treated with 10 μg/mL of AD at 24 h following a switch to 5 mM KCl BME, but there was no significant difference in the survival rates of cells which were treated with either TJ-23 or AD at 48 h following a switch to 5 mM KCl BME. However, differences in the survival rates of cells were found at 72 h following a switch to 5 mM KCl BME; AD treated cells underwent necrosis and only 41% of the cells survived, but TJ-23 treated cells remained healthy and 67% of the cells survived. Moreover, no neuron survived at 96 h after cells were treated with 10 μg/mL of AD, however, more than 50% of neurons survived at 96 h after cells were treated with 0.05 mg/mL of TJ-23 in a serum-free 5 mM KCl BME. From observation made in this study, it seems unlikely that TJ-23 acts like AD in blocking production of killer gene transcription to intervene in apoptosis of neurons.

Therefore, another possible explanation for the intervention of TJ-23 in apoptosis may be considered. Specific neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDGF) which are generated by treatment with TJ-23 may intervene in apoptosis caused by a switch to serum-free 5 mM KCl BME in the cultured cerebellar granule cells. NGF deprivation causes apoptosis in cultured sympathetic neurons and a high concentration of KCl prevents it (Edwards *et al*., 1991). Moreover, NGF and BDGF intervene in apoptosis induced by lowering potassium chloride in the cultured cerebellar granule cells (D'Mello *et al*., 1993; Kubo *et al*., 1995). The neurotrophic factors and high potassium encouraging neuronal survival are thought to increase the concentration of intracellular calcium (Dowd, 1995; Galli *et al*., 1995; De Bernardi *et al*., 1996; Engel *et al*., 1994; Berninger *et al*., 1993). The proliferation of neurons and the expression of c-fos

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oncogene protein need an influx of calcium (Barthel and Loeffer, 1995; Lerea *et al*., 1992) and a sustained increase of steady-state free calcium concentration blocks neuronal apoptosis (Franklin and Johnson, 1994).

Treatment with TJ-23 facilitates the activity of nicotine acetylcholine receptors (Hagino, 1990) and increases intracellular calcium concentrations in the cultured PC12 cells (Hagino, 1996). Our recent study (Hagino, 1996, Zhang, *et al*., 1996) showed that when cerebellar granule cells were cultured in a 25 mM KCl BME containing 10% serum from the beginning, the neuron survival was 89% at 8 DIV. The c-fos protein production, an oncogene protein, was detected with Western blotting and there was no DNA fragmentation. However, when cerebellar granular cells were cultured in 5 mM KCl BME containing 10% serum from the beginning, the neuron survival was only 28% at 8 DIV. There was no c-fos protein production and DNA fragmentation was observed. Addition of 0.05 mg/mL of TJ-23 to 5 mM KCl BME containing 10% serum at 2 DIV, resulted in 65% of neurons alive at 8 DIV. c-fos protein production was detected and there was no DNA fragmentation. Moreover, we observed that addition of 10 μM of MK-801, a blocker of *N*-methy-D-aspartate receptors which blocks calcium flux, at 2 DIV inhibited the anti-apoptosis effect of TJ-23 in the cerebellar granule cells cultured in 5 mM KCl BME; treatment with TJ-23 in the cerebellar granule cells cultured in 5 mM KCl produced the c-fos transcriptional proteins and intervened in DNA fragmentation. However, an additional treatment of MK-801 together with TJ-23 at 2 DIV blocked the production of c-fos proteins and brought about DNA fragmentation at 8 DIV. It is inferred, therefore, that addition of TJ-23 facilitates calcium mobilization in the cerebellar granule cells cultured in 5 mM KCl BME and it intervenes in apoptosis.

In summary, a switch to serum-free 5 mM KCl BME from 25 mM KCl BME with 10% calf serum at 7 DIV induces apoptosis in cultured cerebellar granule cells. Treatment with TJ-23 at 7 DIV intervenes in apoptosis, however, the mode of action of TJ-23 for intervention in apoptosis differs from that of AD.

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