Trigonelline-Induced Neurite Outgrowth in Human Neuroblastoma SK-N-SH Cells

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Extension of dendrites and axons in neurons may compensate and rescue damaged neuronal networks in the dementia brain. Our aim is to isolate and identify constituents of coffee beans exhibiting neurite outgrowth activity. Among the extracts of raw and roasted coffee beans, a methanol fraction of the ethanol extract (1 $\mu\text{g}/\text{ml}$) of raw beans increased significantly the percentage of cells with neurites in human neuroblastoma SK-N-SH cells. Among subfractions of this methanol fraction was a basic fraction (5 $\mu\text{g}/\text{ml}$) which exhibited significant neurite outgrowth activity. Finally, trigonelline in the basic fraction was identified to be active as far neurite extension was concerned. Treatment with trigonelline (30 μM) increased the percentage of cells with neurites at 3 and 6 d after treatment. In addition, the number of neurites reacting positively to phosphorylated neurofilament-H was increased by treatment with 30 μM trigonelline for 6 d, suggesting enhancement of axonal extension. These results show that trigonelline promotes functional neurite outgrowth.

Key words trigonelline; neurite outgrowth; axon; coffee; human neuroblastoma

Several neuropathies, such as Alzheimer's disease, Parkinson's disease and ischemic dementia have their own pathologic characteristics. These diseases result from degeneration and loss of neurons in certain brain areas, such as cholinergic neurons in Alzheimer's disease and dopaminergic neurons in Parkinson's disease. Since division and proliferation of neuronal cells are impossible except in the dentate gyrus¹⁾ in the hippocampus, activating the remaining neurons seems to be one way to regenerate neuronal networks. Synaptic formation in neuronal networks is based on axonal and dendritic structures, and so neurite outgrowth is necessary step and an important index for reconstructing neuronal networks.

Coffee is a universal drink, and is known to stimulate the central nervous system as well as heart and circulation.²⁾ It is thought that these effects are mainly caused by caffeine,³⁾ but the effects of other constituents in coffee on the central nervous system have received very little attention. Coffee beans are one of the crude drugs used in the traditional system of Unani medicine⁴⁾ and examined their biological effects, especially neurite outgrowth activity. In the present paper, we report the effect of various coffee extracts and an active constituent, trigonelline, on neurite outgrowth in human neuroblastoma SK-N-SH cells.

MATERIALS AND METHODS

Preparation of Coffee Extracts Raw and roasted coffee beans from Colombia were powdered in a coffee mill. The degree of roasting is classified by the "lightness" value (*L* value). *L* values of light, medium and deep roasting are as 27.1–30.0, 24.1–27.0 and 18.1–21.0, respectively.⁵⁾ Fifty grams of each powder was extracted with 150 ml water at 95 °C for 30 min (water extract), or 150 ml ethanol under reflux for 1 h (ethanol extract). The ethanol extract was further fractionated with *n*-hexane (EtOH–Hex extract) and 90% methanol (EtOH–MeOH extract). The EtOH–MeOH extract of the raw coffee beans was applied to an anion-exchange column (Amberlite IRA-400, Organo, OH[−] form), and the column was washed with water followed by elution with 1 N

formic acid to give an acidic fraction. The aqueous eluate was then applied to a cation-exchange column (Amberlite IR-120B, Organo, H⁺ form). After washing with water to give a neutral fraction, the column was eluted with aqueous ammonia to give a basic fraction. This basic fraction was then applied to a silica-gel column to give two fractions (Ba-1 and Ba-2). Thin-layer chromatography (TLC) analysis showed that each subfraction, Ba-1 and Ba-2, contained mainly one compound. Co-TLC, ¹H-NMR and ¹³C-NMR experiments comparing the data with that from commercially available standard samples (anhydrous caffeine; Nacalai Tesque, Kyoto, Japan, trigonelline hydrochloride; Tokyo Kasei, Tokyo, Japan) suggested that the major compounds in the Ba-1 and Ba-2 fractions were caffeine and trigonelline, respectively. The NMR data for trigonelline were as follows: ¹H-NMR (D₂O) δ : 4.29 (3H, s), 7.94 (1H, br s), 8.65–8.70 (2H), 8.97 (1H, s); ¹³C-NMR (D₂O) δ : 49.2, 128.6, 137.8, 145.6, 146.7, 146.9, 168.6.

Cell Culture A human neuroblastoma cell line, SK-N-SH (RIKEN, Tsukuba, Japan) was maintained as a monolayer culture in minimum essential medium (GIBCO BRL, Rockville, U.S.A.) supplemented with 5% fetal bovine serum at 37 °C in a humidified atmosphere of 95% air/5% CO₂.

Quantification of Neurite Outgrowth Cells were plated with a plating density of 6.0×10^4 cells/ml in 60-mm diameter culture dishes with 2-mm grids (Corning, New York, U.S.A.). Extracts were applied to the culture medium once, at the start of culture. The vehicle solution was 0.1% dimethyl sulfoxide (DMSO) or distilled water. Cells (100–300 cells) in a fixed area ($675 \times 450 \mu\text{m}$) were observed, and the total value from 4 areas was counted. The percentage of cells with neurites was calculated, only neurites of length greater than 50 μm being counted.

Immunohistochemistry Cells were cultured in 8-chamber slides (Becton Dickinson Labware, New Jersey, U.S.A.) for 6 d in the presence or absence of trigonelline. The slides were then rinsed in phosphate-buffered saline (PBS), fixed with 10% formalin for 30 min at room temperature, and rinsed again with PBS containing 0.2% TritonX-100. Anti-

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Fig. 1. Effects of Extracts from Coffee Beans on Neurite Outgrowth in SK-N-SH Cells

A) Cells were treated with water (Water), EtOH-Hex (Hex) and EtOH-MeOH (MeOH) extracts of raw coffee beans at a final concentration of 1 $\mu\text{g/ml}$ or 0.1% DMSO (cont, open column) for 5 d. B) Cells were also treated with EtOH-MeOH extracts of raw (R), light roasted (L), medium roasted (M) or deep roasted (D) beans at a final concentration of 1 $\mu\text{g/ml}$ or 0.1% DMSO (cont, open column) for 5 d. The percentage of cells with neurites longer than 50 μm is shown. Values represent the data from 4 areas of a dish.

body to phosphorylated neurofilament-H (NF-H, Affiniti Research Products Ltd., Exeter, UK) was used to identify axonal processes.^{6,7} PBS containing 0.3% TritonX-100, monoclonal antibody to NF-H (1:200) and 1% normal donkey serum were added to the fixed slides. After incubation for 1 d at 4°C, slides were rinsed with PBS containing 0.2% TritonX-100 and incubated for 30 min with PBS containing 0.3% TritonX-100 and fluorescein isothiocyanate (FITC)-labeled donkey anti-mouse IgG (1:100). The slides were washed with PBS, mounted using Aqua Poly Mount (Polysciences, Warrington, U.S.A.) and examined under a confocal laser scanning microscope (LSM-GB200-IMT-2, Olympus, Tokyo, Japan).

Data Processing Statistical comparisons were made by Student's *t*-test, one-way analysis of variance or repeated measure two-way analysis of variance (RM-ANOVA); $p < 0.05$ was considered as significant. The means of data are presented together with their S.E.M.

RESULTS

Water, EtOH-Hex and EtOH-MeOH extracts of raw coffee beans were added to the culture medium of SK-N-SH cells at a concentration of 1 $\mu\text{g/ml}$, and neurite outgrowth activity was observed 5 d later (Fig. 1A). Only the EtOH-MeOH extract exhibited neurite outgrowth activity. The neurite outgrowth activity of the EtOH-MeOH extracts from raw beans, light roasted (*L* value=29.8), medium roasted (*L* value=24.5) and deep roasted (*L* value=20.0) beans was compared (Fig. 1B). The more intense the roasting, the

Fig. 2. Effects of Subfractions of EtOH-MeOH Extract on Neurite Outgrowth in SK-N-SH Cells

Cells were treated with EtOH-MeOH extract (Et-Me, hatched column), acidic (Acid), basic (Basic) and neutral (Neut) fractions at a final concentration of 5 $\mu\text{g/ml}$ or 0.1% DMSO (cont, open column) for 5 d. The percentage of cells with neurites longer than 50 μm is shown. Values represent the means and S.E.M. of three dishes. * $p < 0.05$ when compared with control. # $p < 0.05$ when compared with EtOH-MeOH extract.

trigonelline

Chart 1

greater the reduction in neurite outgrowth activity of the extract.

The effects of subfractions of the EtOH-MeOH extract of raw beans were investigated at a concentration of 5 $\mu\text{g/ml}$ in SK-N-SH cells 5 d after administration. The basic fraction significantly increased the ratio of cells with neurites but not the acidic and neutral fractions (Fig. 2). The effect of the basic fraction was more potent than that of the EtOH-MeOH extract itself, under the same conditions and concentration, suggesting that the active constituents are present mainly in the basic fraction. So, the basic fraction was further fractionated to give Ba-1 and Ba-2 as described in the Materials and Methods.

Then, the effects of Ba-1 and Ba-2 were investigated at a concentration of 5 $\mu\text{g/ml}$ in SK-N-SH cells 5 d after administration. Ba-2 significantly increased the ratio of cells with neurites and it was shown on TLC that the Ba-2 fraction contained trigonelline (Chart 1) as a major constituent. Therefore, the activity of Ba-2 was compared with commercial trigonelline (Fig. 3). Trigonelline, at a concentration of 5 $\mu\text{g/ml}$ increased significantly the ratio of cells with neurites. The activity of trigonelline was more potent than that of Ba-2, but the difference was not significant.

We further investigated the neurite outgrowth effect using commercial trigonelline. SK-N-SH cells were treated with trigonelline at 30 μM up to 6 d since the dose of 5 $\mu\text{g/ml}$ used in Fig. 3 corresponded to 28.8 μM trigonelline (Fig. 4). Trigonelline increased significantly the percentage of cells with neurites at 3 and 6 d after treatment. RM-ANOVA analysis revealed a significant trigonelline effect ($F_{1,4}=111.8$, $p < 0.01$) and trigonelline $\times$ time interaction ($F_{3,12}=16.8$, $p < 0.01$). The activity of trigonelline was same at 3 and 6 d. Trigonelline (30 μM) did not increase cell growth

Fig. 3. Effects of Subfractions of Basic Fraction and Trigonelline on Neurite Outgrowth in SK-N-SH Cells

Cells were treated with Ba-1 (closed column), Ba-2 (closed column) and trigonelline (TGN, hatched column) at a final concentration of 5 $\mu\text{g}/\text{ml}$ or 0.1% DMSO (cont, open column) for 5 d. The percentage of cells with neurites longer than 50 μm is shown. Values represent the means and S.E.M. of three dishes. * $p < 0.05$ when compared with control.

Fig. 4. Time-course of the Effect of Trigonelline on Neurite Outgrowth in SK-N-SH Cells

Cells were treated with 30 μM trigonelline (closed circles) or distilled water (open circles). The percentage of cells with neurites longer than 50 μm is plotted against the day after treatment. Values represent the means and S.E.M. of three dishes. * $p < 0.05$ when compared with vehicle.

in the presence of serum nor did it enhance cell survival in the absence of serum (data not shown). Also trigonelline did not also produce any cell toxicity up to a concentration of 100 μM (data not shown).

To investigate the extension of axons, immunohistochemistry for phosphorylated NF-H, an axon marker, was performed in trigonelline-treated SK-N-SH cells. It is known that NF-H is transported from the cell body to the axon, and simultaneously phosphorylation of NF-H occurs.⁸⁾ In actual fact, NF-H-positive signals were observed in cell bodies and several filaments. Long neurites positive for NF-H were more frequent in cells treated with 30 μM trigonelline for 6 d compared with control cells (Fig. 5).

DISCUSSION

We isolated trigonelline from raw coffee beans and found it to be active in promoting neurite outgrowth. Extended neurites induced by trigonelline were axonal, at least in part, suggesting that trigonelline promotes functional neurite outgrowth which may enhance synaptic formation.

It was shown that the neurite outgrowth activity of the EtOH-MeOH extract decreased depending on roasting degree, as shown in Fig. 1B. Trigonelline in coffee beans is known to be converted several compounds including nico-

Fig. 5. Effect of Trigonelline on Expression of Phosphorylated NF-H in SK-N-SH Cells

Cells were treated with 30 μM trigonelline (TGN) or distilled water (cont) for 6 d. Then, fixed cells were immunofluorescent-labeled with anti-phosphorylated NF-H antibody. The fluorescent images are shown in false color; green indicates positive for phosphorylated NF-H, dark blue is negative. Arrows indicate NF-H-positive long neurites. Scale bar = 20 μm .

tinic acid by roasting,^{9,10)} and the reduction in trigonelline is related to the degree of roasting.¹¹⁾ These data support further the hypothesis that the active compound in coffee beans producing neurite outgrowth is trigonelline.

Since no terrestrial animals including Mammalia, have trigonelline-synthesizing enzyme, nicotinate methyltransferase, trigonelline needs to be administered. As regards the effects of exogenous trigonelline, only a hypoglycemic action has been reported.¹²⁾ None of the effects of trigonelline in the neuron or brain has been reported. It is uncertain whether orally administered trigonelline can cross the blood-brain barrier. However, the effect of trigonelline on the brain can be investigated *in vivo* by administration of trigonelline orally or by direct injection into the brain. It would be worthwhile investigating the mechanism of this trigonelline effect on the brain in more detail.

In conclusion, we identified trigonelline as a compound exhibiting neurite outgrowth activity in raw coffee beans, and found that it induced axonal extension in human neuroblastoma cells. The *in vivo* effects of trigonelline on the regeneration of the neuronal network are now under investigation in our laboratory.

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